PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/82, 15/33, 5/10, A01H 5/00

A2

(11) International Publication Number: WO 99/60140

(43) International Publication Date: 25 November 1999 (25.11.99)

(21) International Application Number: PCT/IB99/01282

(22) International Filing Date: 19 May 1999 (19.05.99)

(30) Priority Data:
98201636.2 19 May 1998 (19.05.98) EP

(71) Applicant *(for all designated States except US)*: CENTRO DE INVESTIGACION Y DE ESTUDIOS AVANZADOS DEL I.P.N. (CINVESTAV) [MX/MX]; Departamento de Ingeniería Genética, CP 629, Irapuato, Guanajuato 36500 (MX).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: RIVERA-BUSTAMANTE, Rafael, F. [MX/MX]; Infiernillo 1231, Colonia CFE, Irapuato, Guanajuato 36631 (MX). RUIZ-MEDRANO, Roberto [MX/MX]; Matavacas 5 San Javier, Guanajuato, Guanajuato 36020 (MX). ARGÜELLO-ASTORGA, Gerardo [MX/MX]; Rio Tamesi 1561, Colonia La Pradera, Irapuato, Guanajuato 36630 (MX). MONSALVE-FONNEGRA, Zulma I. [CO/CO]; Calle 49 #46 A100, Barrio San Francisco, Copacabana, Antioquia (CO).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: GEMINIVIRUS INDUCIBLE PROMOTER SEQUENCES AND THE USES THEREOF

(57) Abstract

Novel chimeric promoters which allow controlled transcription and/or expression of a nucleic acid sequence upon geminivirus infection, and the use of such recombinant promoters are provided. Furthermore, recombinant genes comprising such promoters and transgenic plant cells and plants comprising the chimeric promoters or recombinant genes are described.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

Geminivirus inducible promoter sequences and the us s thereof

The present invention relates to promoter sequences which are in themselves – normally, if present in a plant cell, regulated upon geminiviral infection; to the use of those sequences to generate novel recombinant promoters which allow controlled transcription and/or expression of a nucleic acid sequence, and to the use of such recombinant promoters.

It appears that upon infection of the plant with wild-type virus, or a part thereof such as the AC2 protein, expression of adjacent genes occurs under the control and influence of a geminiviral promoter. It appears that small nucleotide sequences, referred to as CLEs (conserved late elements), present in the geminiviral promoter, are sufficient to induce said expression. According to the current invention it is thus feasible to construct transgenic plants, comprising at least one of said CLEs or functional fragments thereof, which are resistant to geminiviral infection. To obtain this effect, adjacent to or operably linked to any of the said CLEs any gene or gene combination can be constructed, which gene or gene product is able to interfere with the outbreak or growth characteristics of the geminivirus in order to arrest further spread of the geminivirus in the infected plant or part thereof.

Background

Viruses are the causative agents of a large number of serious and potentially serious diseases in humans, animals and plants. Plant viruses in particular have the potential to destroy or reduce crop yield and to otherwise have a deleterious effect on agricultural and horticultural industries to economically significant levels. Particularly important viruses in this regard are the DNA viruses, including the geminiviruses.

The geminiviruses are a large and diverse family of plant viruses comprising three genera, Mastre-, Curto- and Begomoviruses. Classification is based on genome structure (mono- or bipartite), natural vector (leafhoppers or white fly species) and host range (mono or dicotyledonous). Mastrevirus are transmitted by leafhoppers and except for a few exceptions infect monocots; their genome comprises a single stranded DNA component. Most Begomoviruses are transmitted by white fly species,

infect dicots and posses a bipartite genome, usually called A and B, of similar sizes. Curtoviruses occupy an intermediate position infecting dicots but with a single stranded DNA genome component. This classification is in accordance with the phylogenetic groups obtained in evolutionary studies.

Examples of Mastrevirus include: Maize Streak Virus (MSV), Digitaria Streak Virus (DSV) and Wheat Dwarf Virus (WDV). Examples of Curtovirus include: Beet Curly Top Virus (BCTV) and Horseradish Curly Top Virus (HCTV). Examples of Begomovirus includes: Bean Golden Mosaic Virus (BGMV), Texas Pepper Geminivirus (TPGV), Squash Leaf Curl Virus (SqLCV), Abutilon Mosaic Virus (AbMV), Ageraturn Yellow Mosaic Virus (AYMV), African Cassava Mosaic Virus (ACMV), Chloris Striate Mosaic Virus (CSMV), Tomato Yellow Leaf Curl Virus (TYLCV), Tomato Golden Mosaic Virus (TGMV) and Tomato Leaf Curl Virus (TLCV). There are many other examples of geminiviruses that can be identified by persons skilled in the art (see, for example, the online viral database VIDE Database (at The Australian National University Bioinformatics Group) at http://biology.anu.edu.au/Groups/MES/vide/genus005.htm) and that are known to cause economically important diseases affecting yield and quality of crops.

Geminiviruses have small circular single stranded DNA genomes, consisting of one or two components, each component being in the range of 2.5 - 3 kb. The coding capacity is low and varies among the different genera, but they all encode proteins in the viral and complementary sense orientation. The coding regions are separated by intergenic regions which are important in replication and transcription of viral DNA. Mastreviruses encode four open reading frames of more that 10 kDa, while Curto and Begomoviruses encode six to seven. There are two nomenclatures for geminiviral genes, (a): V (virion sense) or L (left from 5' intergenic region) if the gene is specified in the virion sense DNA strand or (b): C (complementary) or R (right) if the gene is specified in the complementary sense DNA strand. Thus, C2, AC2 and AL2 refer to homologues ORFs of different viruses. Proteins are usually identified by the name of their gene, that is AC2 protein is encoded by AC2 gene. AC2 protein is also refer as TrAP (transcriptional activator protein).

In bipartite geminiviruses component A codes for all the viral proteins required for replication, transcription and encapsidation of the viral DNA: AC1 (Rep) and AC3, AC2 (TrAP) and the coat protein AV1 (AV2, CP), respectively. The B component codes for the BC1 and BV1 proteins involved in the cell-to-cell and systemic movement and symptom production of the virus in the plant. Both components display an intergenic region (IR) which includes a 180-200 nt segment almost identical in both components and known as the common region. All geminiviruses have an element in this region with the potential to form a stem-loop structure. This entire structure (~30 nt) is conserved among bipartite geminiviruses whereas in monopartite geminiviruses the sequence of the stem varies, conserving only the loop sequence (Lazarowitz, 1992).

Geminiviruses replicate in the nucleus of the infected cell and are thought to employ a rolling-circle mechanism similar to one used by the single-stranded DNA containing coliphages and certain *Staphylococcus aureus* and *Bacillus subtilis* plasmids. The other known plant viruses, including other plant DNA viruses, replicate via RNA intermediates. Geminivirus particles accumulate in the nuclei of infected cells where DNA replication and virus assembly probably take place (Davies et al., 1987). Their putative replicative forms are double-stranded covalently closed circular DNA molecules of about 2,7 Kb folded in chromatine-like structures and are likely to be the transcriptionally active forms of the virus (Abouzid et al., 1988).

Since geminiviruses are the only plant virus group as described above that replicate solely in the nucleus and without RNA intermediates, these features make them amenable for their use as plant expression vectors and as models for the study of plant gene regulation and DNA replication (Davies and Stanley, 1989; Lazarowitz, 1992; Timmermans *et al.*, 1994). The study of gene expression in animal viruses, such as SV40, adenovirus, herpesvirus, among many others, has offered important insights into the corresponding processes in their hosts. Accordingly, the geminiviruses might shed some light into the analogous mechanisms in plant cells.

Upon infection of a host cell by a geminivirus, the viral coat protein is removed and a double stranded replicative form of DNA is synthesized comprising the virion-sense strand and a "complementary-sense" strand. Transcription occurs from both the

virion-sense strand and from the "complementary sense" strand, giving rise to (+) and (-) sense RNA transcripts respectively.

In general, the expression of viral genes follows a temporal sequence that is finely coordinated. At the onset of infection the virus relies entirely on the host transcriptional machinery. However, some of the viral proteins synthesized in the first stage participate in the regulation of viral genes in subsequent stages (reviewed by Martin and Green, 1992; Nevins, 1991). In the case of geminiviruses, it has been reported that Rep downregulates its own expression (Haley et al., 1992: Sunter et al., 1993), whereas AC2 regulates the expression of virion-sense genes from both components, CP and BV1 (Sunter and Bisaro, 1991; Sunter and Bisaro, 1992). The amino acid sequence of AC2 displays features of an eukaryotic transactivator, including a basic domain associated with a typical zinc-finger motif and a carboxy-terminal acidic domain. However, sequence-specific DNA binding of AC2 has not been demonstrated (Noris et al., 1996; Sung and Coutts, 1996).

The intergenic region (IR) of geminiviruses harbors two divergent, overlapping promoters (Lazarowitz, 1992). Even though the upstream limits of these promoters have not been defined, it is likely that most regulatory sequences lie within the IR. The IRs of several geminiviruses have been analyzed in the art and there has been searched for sequences involved in their replication and transcriptional regulation. By means of a phylogenetic-structural approach and based on available experimental evidence, it was postulated that iterative sequences found in this region could function as determinants for replicative specificity of geminiviruses (Argüello-Astorga et al., 1994a; Argüello-Astorga et al., 1994b). Additionally, other sequences that could play a role in the expression of the viral ORFs were also found.

The viron sense promoters are not as well characterized as their complementary-sense counterparts. Transient reporter assays showed that common region DNA and downstream sequences, which contain the putative TATA box and transcription start site can support virion-sense transcription only when AL2 is supplied in trans (Gröning et al., 1994; Sunter et al., 1997). AC2 dependent transcription has also been reported for ACMV (Haley et al., 1992). Recent studies in transgenic plants revealed that regulation of virion-sense transcription is complex. A CP promoter

fragment containing common region DNA and adjacent sequences was subject to AL2-mediated regulation in mesophyll cells. However, the same reporter construct was constitutively expressed in vascular tissue, suggesting that a regulatory element was missing. This element, which was mapped to sequences down stream of the CP gene, negatively regulated expression and conferred AL2 dependence in vascular tissue (Sunter and Bisaro 1997). It had been shown in certain cases that heterologous complementation for the AC2 gene can occur among distantly-related geminiviruses, suggesting that there may be a conservation of the target sequence (Sunter et al., 1994). There was no information regarding the identities of the *cis* elements, including the AL2-responsive element, that contributes to AR1 or BR1 promoter activation.

As mentioned above, geminiviruses cause important diseases in a number of crops. No effective control strategy has been developed to date. Due to the economic importance of plant DNA viruses and, in particular, geminiviruses, there is a need for disease resistance strategies to be developed. Several genetic engineering strategies against viruses have been used based on coat protein expression, however these approaches are not effective against geminiviruses. Other pathogen derived or alternative transgenic resistance strategies (such as expression of toxic genes - Hong et al., 1996) have been explored. Many problems have been encountered such as low level of resistance (Day et al., 1991), and narrow range where resistance was only effective against a few strains of a virus (Frischmuth and Stanley, 1998; Noris, Accotto et al., 1996). Conventional plant breeding programs have provided partial answers in a number of cases, however, frequently its successes are limited, primarily because natural resistance to geminivirus is usually poligenic and most cultivars of a certain crop remain susceptible (Hahn et al., 1980). The potential for an effective control strategy based on a transgenic approach remains very high. However, there has been very little success in obtaining transgenic plants with an enhanced tolerance to geminiviral infection. It is therefore an object of the current invention to propose a solution for this problem.

Description of the present invention

Thus, the technical problem of the present invention is to provide promoters that are rapidly responsive to geminiviral infection but show negligible activity in uninfected

parts of the plant and that can be used for engineering of virus disease resistant crops.

The solution to this technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the invention relates to a chimeric promoter capable of mediating gene expression of a heterologous DNA sequence in plants upon geminivirus infection comprising

(i) at least one cis-acting element sufficient to confer geminivirus-inducible expression comprising the nucleotide sequence of any one of SEQ ID NOS.: 1 to 11 or a functionally equivalent nucleotide sequence; and

(ii) a promoter.

The term "capable of mediating gene expression in plants upon geminivirus infection" as used herein means that said promoter is capable of regulating the transcriptional activation of a heterologous DNA sequence selectively in response to infection of the plant with geminivirus. The transcriptional activation by the chimeric promoter of the invention may preferably occur at the infection site but may also occur in cells surrounding the actual infection site, e.g., due to cell-cell interactions. The chimeric promoter of the invention may advantageously not or only to a small extent be inducible upon other stimuli such as abiotic stress. Preferably, the induction from the chimeric promoter upon geminivirus infection is at least about 2-fold higher, preferably 3-fold higher, particularly preferred 5-fold higher than its activation, if any, by abiotic stress.

The expression specificity conferred by the chimeric promoters of the invention may not be limited to local gene expression due to geminivirus infection, for example, they may have a basal but low expression in the cortex, epidermis and/or stem. In contrast, there is preferably no substantial expression of heterologous DNA sequences under the control of the promoter of the invention in vascular tissue and/or roots in the absence of geminiviral infection. Furthermore, the chimeric promoter of the invention may be combined with further regulatory sequences that provide for tissue specific gene expression. The particular expression pattern may also depend on the plant/vector system employed. However, expression of

heterologous DNA sequences driven by the chimeric promoters of the invention predominantly occurs upon geminivirus infection unless certain elements of the invention were taken and designed by the person skilled in the art to control the expression of a heterologous DNA sequence in certain cell types.

The term "cis-acting element sufficient to confer geminivirus inducible expression" denotes a short stretch of a DNA preferably between 6 and 115 nucleotides in length that when combined with a promoter, preferably minimal promoter such as the CaMV 35S minimal promoter (positions -46 to +8) is capable of directing high level expression of a heterologous DNA sequence in a plant cell in the presence of geminivirus. As will be described below these cis-acting elements are preferably the so-called CLE or CLE-like elements.

It was suggested, in a theoretical study, that there may be some repetitive elements present in the intergenic region of some geminiviruses, either in sense or complementary orientation which may play such a role (Argüello-Astorga, 1994, the relevant figures reproduced in this application in Figure 2 and Figure 5 which are hereby incorporated by reference). These were termed Conserved Late Elements (CLE). The CLE motif is not a sequence commonly found in plant gene promoters. A search among the upstream sequences of plant genes included in the GenBank database reported only a few matches. Interestingly, recent work with the rice PCNA gene promoter demonstrated the functionality of CLE-like sequences (Kosugi *et al.*, 1995). In this work, it was reported that two elements highly homologous to CLEs (TGGTCCC and GTGGGCC), and a G-box were essential for the activity of the promoter in meristems. Thus, it is possible that CLE and CLE-like elements will reveal themselves as regulatory elements in plant promoters. In geminiviral promoters, CLE elements may be found in varying repetitions, relative position and orientation for different viruses. Similarly, sequence and length of those elements can vary between viruses.

For the purposes of the invention described in this application, CLE elements are understood to be cis-acting elements made up of short nucleic acid sequences of approximately 6 – 12 nucleotides in length. A consensus sequence is defined by Argüello-Astorga, 1994 as A/G A/T GTGGTCCC (SEQ ID NO 1). Other examples of

CLEs include: GTGGTCC (SEQ ID NO 2), GTGGTCCA (SEQ ID NO 3), TTGGCCCA (SEQ ID NO 4), TTGGTCCC (SEQ ID NO 5), GTGGACCA (SEQ ID NO 6), GACCAC (SEQ ID NO 7), GGGACCAA (SEQ ID NO 8). These sequences may vary as new variants of viruses are discovered but it is possible to identify such sequences as CLE elements using the method described in Argüello-Astorga, 1994. CLEs may be repeated in varying number, found in different positions relative to one another, in different orientations and different CLEs added together in combination.

The term "promoter", within the meaning of the present invention refers to nucleotide sequences capable of transcription initiation, i.e. RNA polymerase binding, and may also include, for example, the TATA box. Such nucleotide sequences can be derived from naturally occurring plant gene or plant pathogen, e.g., viral promoters, but also from enhancers, inactivated silencer intron sequences, 3'UTR and/or 5'UTR coding regions, RNA stabilizing elements, etc.

By "minimal promoter" it is intended that the basal promoter elements are inactive or nearly so without upstream activation.

The promoters and recombinant genes of this invention described below are further characterized in that substantial nucleotides sequences of the genome of geminivirus is absent. Preferably, the chimeric promoter or recombinant gene of the present invention comprises no more than 150 nucleotides, more preferably no more than 115, still more preferably no more than 50 and particularly preferred, no more than 30 nucleotides of geminivirus sequences. Furthermore, the promoter or recombinant gene of the present invention are preferably characterized by at least one endonuclease recognition site located less than about 150 nucleotides upstream from the transcription and/or translation start site and which is not naturally present in the geminiviral genome. When present in the recombinant gene said restriction endonuclease site may no longer be present in the sense that due to, e.g., ligation of different blunt ends or compatible overhangs generated from different restriction enzymes the original endonuclease recognition site is no longer recognizable. However, the promoter of the present invention in the recombinant gene would still be identifiable due to the presence of non-geminiviral sequences at the previous recognition sites.

The experiments performed in accordance with the present invention demonstrate that the *cis*-acting elements direct geminivirus-induced expression *in vivo*, being active as monomers, multimers and in combination with each other within synthetic promoters. They therefore meet the biotechnological requirements for the engineering of disease resistance. In particular, this application shows that these CLE elements, when placed in the appropriate context, are sufficient to regulate expression of promoters or a part thereof upon geminiviral infection. The mechanism is unknown but presumably CLE elements promote transcription by responding to nuclear factors present and/or activated in a plant cell after geminiviral infection.

This application provides experimental evidence that sense orientation of such CLE sequences, are sufficient to confer responsiveness to geminiviral infection or the AC2 protein. Furthermore, the application provides that the CLE when placed outside of the viral context in combination with a heterologous minimal promoter retains the ability to respond to geminiviral mediated activation. This finding is particularly surprising in the light of the results published suggesting for TGMV where mutation in the CLE motif in the TGMV had no effect on AC2 transactivation (Karkashian, and Maxwell, 1998), together with evidence that other sequences distinct from the CLE elements have been involved in mediating AC2 activation in the cases of BGMV and TGMV (Karkashian and Maxwell 1998; Sunter and Bisaro 1997b; Bisaro, 1998). Although transactivation may occur between related viruses, the extent to which transactivation of a CP promoter by a heterologous virus occurs even within the Begomovirus genera is largely unknown. Some examples where instances of activation of a geminiviral CP promoter by a heterologous virus has been observed include ACMV, TPGV and SqLCV activating the TGMV CP promoter (Sunter et al., 1994). However, TGMV, AbMV and AYMV did not activate the CP promoter of ACMV (Hong et al., 1996).

AC2 mediated activation of CP geminiviral promoters seems to be not universally conserved. There is no evidence of CP promoter activation in Curtovirus (Stanley, 1992; Hormuzdi and Bisaro, 1995). In Mastrevirus the CP promoter is activated by a different mechanism, namely by the Rep protein and not the AC2. No functional homology between the BCTV C2 protein and the Begomovirus TGMV AL2 has been

found (Hormuzdi and Bisaro, 1995). Additionally, BCTV was not able to transactivate a TGMV CP promoter either in transient assays in protoplast or when present in a transgenic plants (Sunter et al., 1994; Sunter and Bisaro, 1997). There is also evidence indicating that the Begomovirus, TYLCV, might lack AC2 mediated transactivation since mutations in the AC2 protein did not affect CP accumulation (Wartig et al., 1997).

In light of the prior art that heterologous viruses and their respective AC2 proteins do not always activate the CP promoter of another geminivirus or have different mechanisms of action, the present invention is particularly surprising, in that a small element within the region of a CP promoter, the CLE element, is capable of broad spectrum heterologous activation. The present application provides experimental evidence for the first time of activation of CLE elements, by heterologous geminiviruses. The application provides examples of a variety of heterologous geminiviruses – some within the same genera, others from different genera and others with very different mechanisms of action for the expression the CP promoter. A particularly surprising example is that of BCTV - which is not even a Begomovirus but a Curtovirus and for which it has been proposed that its own C2 has no role in CP promoter activation (Sunter and Bisaro, 1994; Horzmundi and Bisaro, 1995) - is also capable of activating the CLE elements. These results are also surprising in the light of the fact that there is no evidence for co-operative or redundant gene function in geminiviruses. The invention therefore makes a significant contribution to the art by providing a tool to achieve promoter activation by a wide range of geminiviruses.

It had been shown that a portion of the CP promoter (-163nt) in TGMV was sufficient to induce transactivation (Sunter and Bisaro, 1997). This application provides a refinement to the mapping of that promoter. It has been found that a truncated 115 nucleotide CP promoter sequence (SEQ ID NO.: 9) still remains responsive to a viral transactivator. Transient gene expression and transgenic plant assays demonstrate that said truncated CP promoter sequence is unexpectedly still responsive to a viral transactivator.

The chimeric promoter of the invention may be preferably comprised only of the above defined cis-acting elements and a promoter; i.e. without elements that may

affect the expression pattern of the promoter. As will be discussed below, other regulatory sequences may be added or present dependent on the intended use of the chimeric promoter of the invention. However, preferably the chimeric promoter of the invention lacks elements that interfere with the geminivirus inducible and preferably specific expression and/or which are responsible for the non-selective expression of the promoter the cis-acting element of the invention was derived from. Therefore, said promoter employed for the chimeric promoter of the invention is preferably a truncated or minimal promoter.

The use of a geminiviral inducible promoter responding to a broad range of viruses can help to overcome specificity problems encountered in previous resistance strategies and to develop new ones. The use of small nucleic acid elements such as CLE to regulate expression of a promoter during geminiviral infection is "environmentally friendly" (i.e. an additional advantage in comparison with strategies that use bigger versions of the CP promoter) in the sense that it reduces greatly the possibility of recombination with incoming viruses carrying similar sequences which could give rise to the generation recombinant viruses of unpredicted effect. This is particularly important in geminiviruses as it has been shown that recombination occurs in nature between geminiviral genomes to a high frequency, the intergenic region being a hot spot for recombination, and further that recombination can occur between a transgene and an incoming virus (Frischmuth and Stanley, 1998, and the references incorporated therein).

The invention includes a number of embodiments centered around combinations and permutations of the CLE elements. Such variations may include different numbers of CLEs in the chimeric promoter and/or different types of CLEs in the chimeric promoter. For instance, an oligonucleotide containing two CLE motifs was synthesized (SEQ ID NO.: 10) and characterized in gain-of-function experiments. Transient expression assays showed that a 29 oligonucleotide sequence is still able to confer AC2- responsiveness to heterologous promoters. A smaller oligonucleotide (16 nt) containing a single CLE also conferred this activity (SEQ ID NO.: 11). In addition, when the CLE motifs were mutated in their original context (truncated 115 nt promoter), this modified promoter lost its ability to be transactivated by AC2.

In a preferred embodiment of the invention the chimeric promoter comprises homo- and/or hetero-multimeric forms of said cis-acting element(s); see also the appended Examples. Preferably, said multimeric form is a dimer. Particular preferred are those combinations of cis-acting elements that are described in the Examples and which combination provide for an at least 2-fold, preferably at least 3-fold and particularly preferred at least about 5-fold induction of gene expression in a plant upon infection with geminivirus.

The invention thus provides for chimeric promoters which are activated upon infection by a geminivirus.

Alternatively, activation of the promoter will be obtained in the presence of those viral proteins which are important in activating coat protein expression, preferentially the AC2 protein of any geminivirus. It is therefore part of the invention to use the chimeric promoters according to the invention in order to obtain activation by methods other than direct virus infection, e.g., by means of expressing the AC2 protein in a transgenic plant under the control of a constitutive or a tissue specific promoter. Activation of the chimeric promoter according to the invention will then occur preferentially in those cells in which the AC2 protein is expressed. The expression of the AC2 protein can, for example, be obtained in another plant, and activation could be obtained in the plant obtained as a result of the cross between a plant expressing the AC2 protein and a plant containing the chimeric promoter according to the invention.

The man skilled in the art will be aware of various potential uses of the chimeric promoter according to the invention with a view to exploit its property of geminivirus and AC2 dependent activation of gene expression.

In a preferred embodiment of the chimeric promoter of the invention the truncated or minimal promoter is derived from the CaMV35S promoter, CHS promoter, PR1 promoter, nos promoter, Ac promoter, Adh-1 promoter, Bz1 promoter or LAT52 promoter. Examples of minimal promoters include:

- the Ac encoded transposase minimal promoter (Fridlender, Mol. Gen. Genet. 258(3) (1998), 306-314;

- the truncated maize Adh-1 promoter in pADcat 2 (Ellis, EMBO J. 6 (1987), 11-16);
- the LAT52 minimal promoter required for pollen expression is from -71 to +110 (Twell, Genes and Development 5 (1991), 496-507);
- Bz1 minimal promoter which is obtained from the bronze1 gene of maize (Roth, Plant Cell 3 (1991), 317.

However, other minimal or truncated promoters from other sources may be employed as well.

In a further preferred embodiment of the chimeric promoter of the invention, the distance between said cis-acting element and said minimal promoter is 20 to 100 base pairs. In addition or alternatively, a spacer region preferably composed of 4 to 10 base pairs separates at least two of said cis-acting elements in the chimeric promoter.

In a particularly preferred embodiment of the invention the chimeric promoter comprises at least two of said cis-acting elements comprising the nucleotide sequence of SEQ ID NO: 2 or 10 or a functionally equivalent nucleotide sequence.

In a particularly preferred embodiment of the chimeric promoter of the invention the induction of gene expression upon geminiviral infection is at least 2-fold, preferably at least 3-fold, more preferably 5-fold. The person skilled in the art may employ different chimeric promoters with different background levels and inducibility depending on the intended use. For example, if the approach of coat protein-mediated protection against virus infection is used the chimeric promoter employed may have high background level expression that would not harm the plant and which upon viral infection would increase at high levels such that resistance to the virus can be obtained. The same rational would apply to, e.g., an antisense or ribozyme mediated protection. On the other hand, where the artificial generation of hypersensitive cell death is intended, preferably a chimeric promoter is used that has low or substantially no background activity and that only upon geminivirus infection is activated to an extent that sufficient level of toxic protein is made so as to cause the cell to die. The selection of the appropriate chimeric promoter of the invention depending on its use is well within the skill of the person skilled in the art.

To the scope of this application and with the aim of creating a chimeric promoter regulated by geminiviruses also belongs the use of said geminiviral silencer like elements or any parts or derivatives thereof in combination with the chimeric promoters according to the invention. The combination is likely to be important in certain cases, in which geminivirus independent expression which may occur in certain tissues of transgenic plants according to the invention needs to be avoided.

To the scope of the invention also belongs those variant promoter sequences obtained by modification including for instance exchange of a nucleotide for another nucleotide, inversions, deletions or insertions of a limited number of nucleotides remaining however the same specificity as the sequences described in SEQ ID NOS.: 1-11.

Promoters -optionally derivable from other viruses- with essentially similar sequence as the geminiviral-inducible promoters according to the current invention, which have comparable or identical characteristics, can be isolated from other comparable species using the inventive promoter sequence(s) as, for instance, hybridization probe under conditions known to a skilled person. In another approach the nucleotide sequences or fragments thereof according to the invention can be used by skilled persons as so-called amplification primers in order to isolate promoter fragments with essentially similar sequences from other species by so-called amplification techniques like the PCR method.

Examples of the different possible applications of the chimeric promoter according to the invention as well as its cis-acting elements will be described in detail in the following.

Hence, in a further embodiment, the present invention relates to a recombinant gene comprising the above-described chimeric promoter. Preferably, the recombinant gene is configured such that the chimeric promoter is operatively linked to a heterologous DNA sequence.

The term "heterologous" with respect to the DNA sequence being operatively linked to the chimeric promoter of the invention means that said DNA sequence is not naturally linked to the chimeric promoter of the invention.

The term "operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. The chimeric promoter "operably linked" to a heterologous DNA sequence is ligated in such a way that expression of a coding sequence is achieved under conditions compatible with the control sequences. Expression comprises transcription of the heterologous DNA sequence preferably into a translatable mRNA. Regulatory elements ensuring expression in eukaryotic, i.e. plant cells are well known to those skilled in the art. In the case of eukaryotic cells they comprise optionally poly-A signals ensuring termination of transcription and stabilization of the transcript, for example, those of the 35S RNA from Cauliflower Mosaic Virus (CaMV) and the Nopaline Synthase gene from Agrobacterium tumefaciens. Additional regulatory elements may include transcriptional as well as translational enhancers. A plant translational enhancer often used is the CAMV omega sequences, the inclusion of an intron (Intron-1 from the Shrunken gene of maize, for example) has been shown to increase expression levels by up to 100-fold. (Mait, Transgenic Research 6 (1997), 143-156; Ni, Plant Journal 7 (1995), 661-676).

It is known in the art that AC2 independent expression of the viral coat protein promoter may occur in certain tissues and also that such AC2 independent expression can be silenced in the presence of another regulatory element of the viral genome called the silencer like element.

The CLEs are sufficient to get transactivation by AC2. By the introduction of promoters (either the wild type CP promoter or a synthetic promoter containing one or more CLEs) into a plant (transgenic or transient experiments) detection of transactivation in these plants is feasible when AC2 is present. However it appears that in some tissues there are plant cellular proteins present which could act like AC2 and transactivate accordingly. With a silencer element the host proteins will not transactivate and the promoter will be turned on only when the AC2 protein is present. A "silencer" is understood as a promoter element that negatively regulates the transcription from the promoter. If the promoter is deleted the transcription is up-regulated inappropriately. Therefore the "silencer" is necessary for the correct activation of the promoter.

The above was observed with a construct that has been made containing a copy of the component A (2631nt) as described in Figure 1A of Torres-Pacheco et al., (1993) and the 115 nt CP promoter (SEQ ID NO.: 9). So a construct was obtained of 2746 bases (2631 and 115) wherein the 115 nt fragment was actually duplicated (present at both ends). Said *silencer-like* element is located around nt position number 2150 according to the sequence numbering in Figure 1A of Torres-Pacheco et al., 1993.

Such a silencer element can also be found in other geminiviruses, e.g., in TGMV between nt –1248 and – 1552 of the whole CP promoter, (Sunter and Bisaro, 1997). Due to the similarities in structural and genome organization in geminiviruses it can be assumed that functional similar elements can be found in other geminiviruses in the same relative position.

To the scope of this application and with the aim of creating a chimeric promoter regulated by geminiviruses also belongs the use of said geminiviral silencer like elements or any parts or derivatives thereof in combination with the chimeric promoters according to the invention. The combination is likely to be important in certain cases, in which geminivirus independent expression which may occur in certain tissues of transgenic plants according to the invention needs to be avoided.

In this respect, it should be noted that in one embodiment of the recombinant gene of the invention at least one of said cis-acting elements or silencer like elements is located in the 5'- or 3-untranslated region or in an intron of the recombinant gene.

The present invention may be used for the overproduction of heterologous genes in plants (see Ma et al., 1996, Cramer et al., 1996; Muhlback, 1998 for examples of pharmaceutical production in plants) and bacterial cultures. There is evidence that the CP promoter is active in prokaryotes (Petty et al., 1986) and it has been shown that geminiviruses can replicate in agrobacterium (Rigden et al., 1996). In principle these geminiviral promoters could be used to overexpress various pharmaceutically important polypeptides in prokaryotes and plants. In order to show that such a system is active in agrobacterium for instance, a vector containing 2 CLEs fused to a minimum 35S promoter to drive expression of an uid A gene (GUS) together with a

polyadenylation signal (e.g., of the 35S gene) would be transformed into Agrobacterium. The Agrobacterium would be cultured to allow expression, cells collected and an assay for GUS expression performed (Vancanneyt et al., 1990).

Thus, in a preferred embodiment, the present invention relates to the recombinant gene of the invention wherein said heterologous DNA sequence encodes a (poly)peptide, cytotoxic protein, antibody, antisense RNA, sense RNA, ribozyme or transcription factor.

The recombinant gene of the invention can be used alone or as part of a vector to express heterologous DNA sequences, which, e.g., encode proteins for, e.g., the control of disease resistance or diagnostics of geminivirus inducible or related gene expression. The recombinant gene or vector containing the DNA sequence encoding an RNA or a protein of interest is introduced into the cells which in turn produce the RNA or protein of interest. For example, the chimeric promoter of the invention can be operatively linked to DNA sequences encoding Barnase for use in the production of localized cell death in plants upon pathogen attack.
On the other hand, said protein can be a scorable marker, e.g., luciferase, green fluorescent protein or ß-galactosidase. This embodiment is particularly useful for the early diagnosis of geminivirus attack of crop plant in a field.
The chimeric promoters of the invention may also be used in methods of antisense approaches. The antisense RNA may be a short (generally at least 10, preferably at least 14 nucleotides, and optionally up to 100 or more nucleotides) nucleotide sequence formulated to be complementary to a portion of a specific mRNA sequence and/or DNA sequence of the gene of interest. Standard methods relating to antisense technology have been described; see, e.g., Klann, Plant Physiol. 112 (1996), 1321-1330. Following transcription of the DNA sequence into antisense RNA, the antisense RNA binds to its target sequence within a cell, thereby inhibiting translation of the mRNA and down-regulating expression of the protein encoded by the mRNA.

Furthermore, appropriate ribozymes can be employed (see, e.g., EP-A1 0 291 533, EP-A1 0 321 201, EP-A2 0 360 257) which specifically cleave the (pre)-mRNA of a target gene. Selection of appropriate target sites and corresponding ribozymes can be done as described for example in Steinecke, Ribozymes, Methods in Cell Biology

50, Galbraith, eds Academic Press, Inc. (1995), 449-460. Further applications of the chimeric promoter are evident to the person skilled in the art and can be derived from the literature, e.g., Strittmatter and Wegener, Zeitschrift für Naturforschung 48c (1993), 673-688; Kahl, J. Microbiol. Biotechnol. 11 (1995), 449-460 and references cited therein.

Said transcription factor can for example be a master regulatory factor that controls the expression of a cascade of genes involved in pathogen defense of the plant (Grotewold, Plant Cell 10 (1998), 721-740).

Cytotoxic proteins comprise, for example, plant RIPs (ribosome inactivating proteins; (Stripe, Bio/Technology 10 (1992), 405-412), defensins (Broekaert, Plant Physiol. 108 (1995), 1353-1358), Bt toxin, α-amylase inhibitor, T4-lysozyme, avirulence gene products, or enzymes such as glucose oxidase which generate reactive oxygen species (Shah, Trends Biotechnol. 13 (1995), 362-368; Shah, Curr. Opin. Biotech. 8 (1997), 208-214; Beachy, Curr. Opin. Biotech. 8 (1997), 215-220; Cornelissen, Plant Physiol. 101 (1993), 709-712; Estruch, Nucleic Acids Res. 22 (1994), 3983-3989).

Furthermore, functional (poly)polypeptides can be expressed such as enzymes, antigens useful in vaccination, feed and food supplements, etc., or mutant forms of such (poly)polypeptides for various purposes.

It is in principle possible to modify the coding sequence in such a way that the protein is located in any desired compartment of the plant cell. These include the nucleus, endoplasmatic reticulum, the vacuole, the mitochondria, the plastids, the apoplast, the cytoplasm etc. Methods how to carry out this modifications and signal sequences ensuring localization in a desired compartment are well known to the person skilled in the art. (Görlich, Science 271 (1996), 1513-1518; Hicks, Plant Physiol. 107 (1995), 1055-1058; Rachubinski, Cell 83 (1995), 525-528; Schatz, Science 271 (1996), 1519-1526; Schnell, Cell 83 (1995), 521-524; Verner, Science 241 (1988), 1307-1313; Vitale, BioAssays 14 (1992), 151-160).

The present invention also relates to vectors, particularly plasmids, cosmids, viruses and bacteriophages used conventionally in genetic engineering that comprise a chimeric promoter or a recombinant gene of the invention. Preferably, said vector is a

plant expression vector, preferably further comprising a selection marker for plants. For example of suitable selector markers, see supra. Methods which are well known to those skilled in the art can be used to construct recombinant vectors; see, for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989), (1994). Alternatively, the chimeric promoters and recombinant genes of the invention can be reconstituted into liposomes for delivery to target cells.

Advantageously, the above-described vectors of the invention comprise a selectable and/or scorable marker. Selectable marker genes useful for the selection of transformed plant cells, callus, plant tissue and plants are well known to those skilled in the art and comprise, for example, antimetabolite resistance as the basis of selection for dhfr, which confers resistance to methotrexate (Reiss, Plant Physiol. (Life Sci. Adv.) 13 (1994), 143-149); npt, which confers resistance to the aminoglycosides neomycin, kanamycin and paromycin (Herrera-Estrella, EMBO J. 2 (1983), 987-995) and hygro, which confers resistance to hygromycin (Marsh, Gene 32 (1984), 481-485). Additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman, Proc. Natl. Acad. Sci. USA 85 (1988), 8047); mannose-6-phosphate isomerase which allows cells to utilize mannose (WO 94/20627) and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.) or deaminase from Aspergillus terreus which confers resistance to Blasticidin S (Tamura, Biosci. Biotechnol. Biochem. 59 (1995), 2336-2338).

Useful scorable marker are also known to those skilled in the art and are commercially available. Advantageously, said marker is a gene encoding luciferase (Giacomin, Pl. Sci. 116 (1996), 59-72; Scikantha, J. Bact. 178 (1996), 121), green fluorescent protein (Gerdes, FEBS Lett. 389 (1996), 44-47) or ß-glucuronidase (Jefferson, EMBO J. 6 (1987), 3901-3907). This embodiment is particularly useful for simple and rapid screening of cells, tissues and plants containing a vector of the invention.

The present invention furthermore relates to host cells comprising a chimeric promoter, recombinant gene or a vector according to the invention wherein the chimeric promoter is foreign to the host cell.

By "foreign" it is meant that the chimeric promoter is either heterologous with respect to the host cell, this means derived from a cell or organism with a different genomic background, or is homologous with respect to the host cell but located in a different genomic environment than the naturally occurring counterpart of said cis-acting element. This means that, if the cis-acting element is homologous with respect to the host cell, it is not located in its natural location in the genome of said host cell, in particular it is surrounded by different genes. The vector or recombinant gene according to the invention which is present in the host cell may either be integrated into the genome of the host cell or it may be maintained in some form extrachromosomally. The host cell can be any prokaryotic or eukaryotic cell, such as bacterial, insect, fungal, plant or animal cells. Preferred cells are plant cells.

In a further preferred embodiment, the present invention provides for a method for the production of transgenic plants, with a reduced susceptibility to a geminiviral infection comprising the introduction of a chimeric promoter, recombinant gene or vector of the invention into the genome of a plant, plant cell or plant tissue. For the expression of the heterologous DNA sequence under the control of the chimeric promoter according to the invention in plant cells, further regulatory sequences such as poly A tail may be fused, preferably 3' to the heterologous DNA sequence, see also supra. Further possibilities might be to add Matrix Attachment Sites at the borders of the transgene to act as "delimiters" and insulate against methylation spread from nearby heterochromatic sequences.

Methods for the introduction of foreign genes into plants are also well known in the art. These include, for example, the transformation of plant cells or tissues with T-DNA using Agrobacterium tumefaciens or Agrobacterium rhizogenes, the fusion of protoplasts, direct gene transfer (see, e.g., EP-A 164 575), injection, electroporation, vacuum infiltration, biolistic methods like particle bombardment, pollen-mediated transformation, plant RNA virus-mediated transformation, liposome-mediated transformation, transformation using wounded or enzyme-degraded immature embryos, or wounded or enzyme-degraded embryogenic callus and other methods

known in the art. The vectors used in the method of the invention may contain further functional elements, for example "left border"- and "right border"-sequences of the T-DNA of Agrobacterium which allow stable integration into the plant genome. Furthermore, methods and vectors are known to the person skilled in the art which permit the generation of marker free transgenic plants, i.e. the selectable or scorable marker gene is lost at a certain stage of plant development or plant breeding. This can be achieved by, for example cotransformation (Lyznik, Plant Mol. Biol. 13 (1989), 151-161; Peng, Plant Mol. Biol. 27 (1995), 91-104) and/or by using systems which utilize enzymes capable of promoting homologous recombination in plants (see, e.g., WO97/08331; Bayley, Plant Mol. Biol. 18 (1992), 353-361); Lloyd, Mol. Gen. Genet. 242 (1994), 653-657; Maeser, Mol. Gen. Genet. 230 (1991), 170-176; Onouchi, Nucl. Acids Res. 19 (1991), 6373-6378). Methods for the preparation of appropriate vectors are described by, e.g., Sambrook (Molecular Cloning; A Laboratory Manual, 2nd Edition (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Suitable strains of Agrobacterium tumefaciens and vectors as well as transformation of Agrobacteria and appropriate growth and selection media are well known to those skilled in the art and are described in the prior art (GV3101 (pMK90RK), Koncz, Mol. Gen. Genet. 204 (1986), 383-396; C58C1 (pGV 3850kan), Deblaere, Nucl. Acid Res. 13 (1985), 4777; Bevan, Nucleic. Acid Res. 12(1984), 8711; Koncz, Proc. Natl. Acad. Sci. USA 86 (1989), 8467-8471; Koncz, Plant Mol. Biol. 20 (1992), 963-976; Koncz, Specialized vectors for gene tagging and expression studies. In: Plant Molecular Biology Manual Vol. 2, Gelvin and Schilperoort (Eds.), Dordrecht, The Netherlands: Kluwer Academic Publ. (1994), 1-22; EP-A-120 516; Hoekema: The Binary Plant Vector System, Offsetdrukkerij Kanters B.V., Alblasserdam (1985), Chapter V, Fraley, Crit. Rev. Plant. Sci., 4, 1-46; An, EMBO J. 4 (1985), 277-287). Although the use of Agrobacterium tumefaciens is preferred in the method of the invention, other Agrobacterium strains, such as Agrobacterium rhizogenes, may be used, for example if a phenotype conferred by said strain is desired.

Methods for the transformation using biolistic methods are well known to the person skilled in the art; see, e.g., Wan, Plant Physiol. 104 (1994), 37-48; Vasil, Bio/Technology 11 (1993), 1553-1558 and Christou (1996) Trends in Plant Science 1, 423-431. Microinjection can be performed as described in Potrykus and Spangenberg (eds.), Gene Transfer To Plants. Springer Verlag, Berlin, NY (1995).

The transformation of most dicotyledonous plants is possible with the methods described above. But also for the transformation of monocotyledonous plants several successful transformation techniques have been developed. These include the transformation using biolistic methods as, e.g., described above as well as protoplast transformation, electroporation of partially permeabilized cells, introduction of DNA using glass fibers, etc.
The resulting transformed plant cell can then be used to regenerate a transformed plant in a manner known by a skilled person.

Alternatively, a plant cell can be used and modified such that said plant cell expresses an endogenous gene under the control of the chimeric promoter. The introduction of the chimeric promoter of the invention which does not naturally control the expression of a given gene or genomic sequences using, e.g., gene targeting vectors can be done according to standard methods, see supra and, e.g., Hayashi, Science 258 (1992), 1350-1353; Fritze and Walden, Gene activation by T-DNA tagging. In *Methods in Molecular* biology 44 (Gartland, K.M.A. and Davey, M.R., eds). Totowa: Human Press (1995), 281-294) or transposon tagging (Chandlee, Physiologia Plantarum 78 (1990), 105-115).

Said plants can be used to study or monitor geminiviral infection processes, either with a view to improve our understanding of transcription regulation or with a view to detect and measure the degree of viral infection in greenhouse or field conditions.

As mentioned herein before, the recombinant gene of the present invention preferably comprises further DNA sequences, preferably gene coding sequences under the control of the promoter of the invention. Said other gene may also be a gene, of either plant origin or non-plant origin, that reduces the viability or the metabolic activity or kills the plant cell in which it is expressed (e.g. by using a gene such as bacterial barnase which is well known in the art or any other gene which is cytotoxic or inhibits normal cellular metabolism). In this way and following the method of transforming plants according to the invention, transgenic plants according to the invention will be obtained which exhibit reduced susceptibility to the geminivirus. Said other gene may also be a gene to be produced in a plant with a view to produce a certain heterologous protein in plants, e.g., a protein with therapeutic utility. The

production of this protein can then be controlled by geminivirus infection, e.g. by means of infection with a geminivirus that does not cause any symptoms of pathogenesis or by means of the induction with ectopically expressed AC2 as described hereinabove.

In general, the plants which can be modified according to the invention can be derived from any desired plant species. They can be monocotyledonous plants or dicotyledonous plants, preferably they belong to plant species of interest in agriculture, wood culture or horticulture interest, such as crop plants (e.g. maize, rice, barley, wheat, rye, oats etc.), potatoes, oil producing plants (e.g. oilseed rape, sunflower, pea nut, soy bean, etc.), cotton, sugar beet, sugar cane, leguminous plants (e.g. beans, peas etc.), wood producing plants, preferably trees, etc.

Thus, the present invention relates also to transgenic plant cells comprising, preferably stably integrated into the genome, a chimeric promoter, a recombinant gene or vector according to the invention or obtainable by the above-described method.

Furthermore, the present invention also relates to transgenic plants and plant tissue comprising the above-described transgenic plant cells or obtainable by the above-described method. These plants may show, for example, increased disease resistance.

In a preferred embodiment of the invention, the transgenic plant upon the presence of the chimeric promoter or the recombinant gene of the invention attained resistance or improved resistance against a geminivirus the corresponding wild-type plant was susceptible to.

The term "resistance" covers the range of protection from a delay to complete inhibition of disease development.

In yet another aspect the invention also relates to harvestable parts and to propagation material of the transgenic plants according to the invention which contain transgenic plant cells described above. Harvestable parts can be in principle any

useful part of a plant, for example, leaves, stems, fruit, seeds, roots, flours, pollen, etc. Propagation material includes, for example, seeds, fruits, cuttings, seedlings, tubers, rootstocks, etc.

In addition, the present invention relates to a kit comprising the chimeric promoter, the recombinant gene, or the vector of the invention. The kit of the invention may contain further ingredients such as selection markers and components for selective media suitable for the generation of transgenic plant cells, plant tissue or plants. Furthermore, the kit may include buffers and substrates for reporter genes that may be present in the recombinant gene or vector of the invention. The kit of the invention may advantageously be used for carrying out the method of the invention and could be, inter alia, employed in a variety of applications referred to herein, e.g., in the diagnostic field or as research tool. The parts of the kit of the invention can be packaged individually in vials or in combination in containers or multicontainer units. Manufacture of the kit follows preferably standard procedures which are known to the person skilled in the art. The kit or its ingredients according to the invention can be used in plant cell and plant tissue cultures. The kit of the invention and its ingredients are expected to be very useful in breeding new varieties of, for example, plants which display improved properties such as virus resistance.
It is also immediately evident to the person skilled in the art that the chimeric promoters, recombinant genes and vectors of the present invention can be employed to produce transgenic plants with a desired trait (see for review TIPTEC Plant Product & Crop Biotechnology 13 (1995), 312-397) comprising production systems of heterologous proteins, e.g., therapeutic proteins or biopolymers in plants.

The present invention for the first time demonstrates that a number of cis-acting elements that are responsible for inducibility of geminiviral genes can be used either alone or in combination with themselves or with other cis-acting elements to construct chimeric promoters that are capable of mediating highly inducible gene expression in plants upon geminivirus attack.

Thus, the present invention relates to the use of a cis-acting element sufficient to direct geminivirus inducible gene expression and in particular to the use of the

chimeric promoter, the recombinant gene, the vector, and the cis-acting elements of the present invention for the production of geminivirus resistant plants.

In a still further embodiment, the present invention relates to a method of rendering a gene responsive to geminivirus infection comprising inserting at least one cis-acting element sufficient to direct geminivirus inducible expression into the promoter of said gene. As is evident to the person skilled in the art a promoter that displays the capabilities of the chimeric promoter of the invention can also be obtained by introducing the cis-acting element as defined above into a promoter of a gene, preferably in close proximity to the transcription and/or translation initiation site of the gene.

In another embodiment, the present invention relates to a method for preparing a promoter capable of mediating gene expression in plants upon geminivirus infection comprising operably linking a cis-acting element sufficient to confer geminivirus inducible expression to a transcription initiation sequence of a promoter. Preferably, said cis-acting element to be inserted in the above-described methods is a cis-acting element of the present invention or as defined in the foregoing embodiments or a multimeric form thereof as defined hereinabove.

In a preferred embodiment of the invention, the above-described methods further comprising deleting non-specific cis-acting elements in the promoter; see supra. Introduction of the cis-acting element of the invention into a given promoter per se may not be sufficient to direct the promoter to exclusively mediate local gene expression in plants upon geminivirus infection. In this case, preexisting elements that may be responsive, for example, to light, hormones, low temperatures, drought or salt stress are preferably deleted.

The above described methods give rise to novel chimeric promoters that are at least partially, preferably fully controlled by geminivirus infection.

Accordingly, the present invention also relates to the promoter obtainable by a method as described above. Said promoter can then be employed, e.g., for the embodiments described hereinabove.

Another aspect of the invention is a method for preventing or diminishing geminivirus infection in a plant and subsequent outgrowth of said virus comprising growing a plant according to the invention in an area susceptible to geminiviral infection.

Another aspect of the current invention is the use of said promoter region comprising any of said DNA sequences to specifically arrest the spread or outgrowth of geminivirus in an infected plant as a result of killing or damaging the geminiviral infected cells.

These and other embodiments are disclosed and encompassed by the description and examples of the present invention. Further literature concerning any one of the methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries, using for example electronic devices. For example the public database "Medline" may be utilized which is available on the Internet, for example under http://www.ncbi.nlm.nih.gov/PubMed/medline.html. Further databases and addresses, such as http://www.ncbi.nlm.nih.gov/, http://www.infobiogen.fr/, http://www.fmi.ch/biology/research_tools.html, http://www.tigr.org/, are known to the person skilled in the art and can also be obtained using, e.g., http://www.lycos.com. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

In order to clarify what is meant in this description by some terms a further explanation is hereunder given.

The terms “polynucleotide”, “DNA sequence”, ”nucleic acid sequence” or “nucleotide sequence” as used herein refers to a polymeric form of nucleotides of any length. This term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA. It also includes known types of modifications, for example, methylation, “caps” substitution of one or more of the naturally occurring nucleotides with analog.

"Transformation" as used herein, refers to the transfer of an exogenous polynucleotide into a host cell, irrespective of the method used for the transfer. The polynucleotide may be transiently or stably introduced into the host cell and may be maintained non-integrated, for example, as a plasmid, or alternatively, may be integrated into the host genome. Many types of vectors can be used to transform a plant cell and many methods to transform plants are available. Examples are direct gene transfer, pollen-mediated transformation, plant RNA virus-mediated transformation, *Agrobacterium*-mediated transformation, liposome mediated transformation, transformation using wounded or enzyme-degraded immature embryos, or wounded or enzyme-degraded embryogenic callus. All these methods and several more are known to persons skilled in the art. The resulting transformed plant cell can then be used to regenerate a transformed plant in a manner known by a skilled person.

"Functional part or fragment thereof" means that said part or fragment to which subject it relates has substantially the same activity as the subject itself, although the form, length or structure may vary.

With "recombinant DNA" or "chimeric DNA" is meant a hybrid DNA produced by joining pieces of DNA from different sources.

In the current invention is meant by "foreign DNA, foreign sequence or foreign gene" , a DNA sequence which is not in the same genomic environment (e.g. not operably linked to the same promoter and/or 3' end) in a plant cell, transformed with said DNA according to said invention, as is such DNA when it naturally occurs in a plant cell or the organism (bacterium, fungi, virus or the like) from which the DNA originates.

"Plant cell" comprises any cell derived from any plant and existing in culture as a single cell, a group of cells or a callus. A plant cell may also be any cell in a developing or mature plant in culture or growing in nature.

"Plants" comprises all plants, including monocotyledonous and dicotyledonous plants.

"Expression" means the production of a protein or nucleotide sequence in the cell itself or in a cell-free system. It includes transcription into an RNA product, post-transcriptional modification and/or translation to a protein product or polypeptide from a DNA encoding that product, as well as possible post-translational modifications.

A more detailed description of the invention, for the sake of clarity, is disclosed hereafter.

The Figures show:

Figure 1. Delimitation of the regions required for AC2-mediated transactivation. Schematic representation of the CP promoter versions used for stable plant transformation. Three versions of the PHV CP promoter were obtained by digesting with different enzymes. The size of each promoter is given as the distance between the 5' end and the CP start codon. (E = *Eco*RI, P = *Pml*I, H = *Hin*dIII, St = *Sty*I, S-L = Stem-loop structure. The three versions were translationally fused to the *Escherichia coli* ß-glucuronidase (*uid*A; GUS) coding sequence and the *nos* terminator.

Figure 2. Location and orientation of CLE sequences in selected geminiviruses.
CLEs found in CP/AV2 and BV1 gene promoters from selected OW (TYLCV-Th, ACMV-N, and ICMV) and NW (PHV, SLCV-E and TGMV) geminiviruses. Filled-in boxes represent elements with the consensus sequence, whereas hatched boxes represent elements with one base mismatch. Relative orientation of the CLEs is indicated by the arrows.

Figure 3. CLE-containing heterologous promoters used in transient expression experiments.
To determine the functionality of CLEs, oligonucleotides containing two CLEs were synthesized and cloned upstream of the minimal (-46/+8) and truncated (-90/+1) versions of the cauliflower mosaic virus (CaMV) 35S promoter to direct the expression of the reporter gene (GUS). The CLE sequences are boxed and the changes introduced to generate a *Stu*I site are shown in lower case.

Figure 4. Quantitative analysis of CLE-mediated transactivation in tobacco leaves.
The constructs CLE46GUS, 1CLE46GUS, and 46GUS (control without CLE sequences) were co-bombarded into tobacco leaf tissue with two sources of AC2 (PHV A and 35S-AC2 DNAs). As controls, pBluescript DNA (BS), and DNA from a AC2 non-expressing PHV A mutant (AC2-) were also used. The number of blue spots in a given area were counted and plotted. Bars represent the average of the values obtained in three experiments; closed circles represent the values obtained in the individual experiments.
A similar experiment was carried out with the constructs 90GUS (control), CLE90GUS, and 1CLE90GUS.

Figure 5. A CLE-less PHV CP promoter is not transactivated.
To verify the importance of the CLE motifs in the original context during the transactivation process, a CLE and two inverted CLE-like motifs were mutated. Wild type and CLE-less SCP-GUS constructs were co-bombarded into tobacco tissue in similar experiments as the one explained in Figure 4.

The present invention is further described by reference to the following non- limiting figures and examples.

Unless stated otherwise in the Examples, all recombinant DNA techniques are performed according to protocols as described in Sambrook et al. (1989), Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, NY or in Volumes 1 and 2 of Ausubel et al. (1994), Current Protocols in Molecular Biology, Current Protocols. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfase (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd. (UK) and Blackwell Scientific Publications (UK).

Examples and description of materials and methods used

Summary of the Examples

Transgenic tobacco plants harboring a construct with 115 nt of the CP promoter (SCP) fused to a GUS reporter gene according to the invention displayed blue spots

consistently at the sites of impact when bombarded with PHV DNA (pepper huasteco virus). These plants normally do not express the transgene in most tissues. These results show that the *cis* acting elements involved in the transactivation process are located within the 115 bases upstream of the CP start codon, a region containing one CLE and two CLE-like motifs. To confirm that the CLEs are indeed responsible for the observed transactivation two other approaches were followed. First, the CLE motifs in the CP promoter were mutated. Although the modified promoter was still able to direct the expression of a reporter gene, no AC2-mediated transactivation was observed. Since one CLE and two inverted CLE-like sequences were all mutated, it is not possible to verify if only one or all of them are required for transactivation. In the second approach, a synthetic oligonucleotide that contains two perfect CLE motifs, conferred AC2-responsiveness to heterologous promoters. A smaller oligonucleotide (16 nt) that includes only a single CLE, was also able to mediate the transactivating effect of AC2 in the context of heterologous promoters. Since the CLE sequence is the only common motif shared between the 16 nt segment and the AC2-responsive truncated -115 CP promoter, therefore said element is the functional core-sequence involved in the transactivation process. A single CLE motif replacing an I-box element in a 52 nt region from the tobacco *rbcS* gene promoter, including the photoresponsive I-G unit (Argüello-Astorga and Herrera-Estrella, 1995), conferred AC2-responsiveness to a chimeric rbcS-35S promoter as determined by transient expression assays in pea leaf tissue.

Although the transactivation effect was observed with both AC2 sources (e.g., whole virus or an AC2-expressing cassette), a stronger expression is usually observed with the whole virus, even when the AC2 gene copy number delivered was similar. On one hand, it is possible that the virus delivered on the target cells can start replication, and thus, increase the AC2 gene copy number. However, other viral factors can also be directly or indirectly affecting CP expression. All CLE-less constructs show a lower expression than its equivalent CLE containing construct (e.g., 46GUS vs CLE46GUS). Nevertheless, the more profound difference in the expression levels obtained with the wt virus and the AC2-mutant agree with the fact that the AC2-mediated transactivation is the main mechanism for the increased expression of the reporter gene.

The 25 amino acid residues of the zinc finger-like domain is the most conserved protein segment of AC2 in subgroup III (SIII) geminiviruses, and it is also conserved in two subgroup II (SII) viruses, namely, the leafhopper-transmitted BCTV-CFH (but not the Logan, California and Worland isolates), and the treehopper-transmitted tomato pseudocurly top virus (TPCTV; Briddon *et al.*, 1996). These are the only known SII geminiviruses that have CLE sequences. It has to be mentioned, however, that C2 in BCTV-Logan is not necessary for coat protein expression, suggesting a different role for this protein.

Sequence analysis

Sequences were assembled and analyzed using GeneWorks (Intelligenetics Inc.) and Lasergene (DNAStar) software packages. The following sequences were obtained from the EMBL and GenBank databases : African cassava mosaic virus Kenya (ACMV-K; accession num. X17095, X17096) and Malawi isolates (ACMV-M) (Hong *et al.*, 1993); Abutilon mosaic virus (AbMV; X15983, X15984); beet curly top virus (BCTV; X04144); bean golden mosaic virus, Puerto Rico (BGMV-PR; D00200, D00201), Brazil (BGMV-BZ; M88686, M88687), and Guatemala isolates (BGMV-GA; M91604, M91605); bean dwarf mosaic virus (BDMV; M88178, M88179); Indian cassava mosaic virus (ICMV; Z24758, Z24759); mung bean yellow mosaic virus (MYMV; D14703, D14704); pepper huasteco virus (PHV; X70418, X70419); Texas pepper virus (TPV; U57457); potato yellow mosaic virus (PYMV; D00940, D00941); squash leaf curl virus (SLCV; M38182, M38183); tomato golden mosaic virus (TGMV; K02029, M73794); tomato mottle virus (TMoV; L14460, L14461); tomato leaf curl virus, Indian (ToLCV-In; L12739) and Australian isolates (ToLCV-Au; Dry *et al.*, 1993); tomato yellow leaf curl virus, Israel (TYLCV-Is; X15656), Sardinia (TYLCV-Sr; X61153), and Thailand isolates (TYLCV-Th; M59838, M59839).

Vector construction

All DNA techniques were carried out according to standard procedures (Sambrook *et al.*, 1989) or as recommended by the suppliers. Full length clones of PHV (pepper huasteco virus) A component in pBluescript SK+ were obtained using either *Eco*RI (pIGV23) or *Hin*dIII (pIGV22) restriction enzymes (Torres-Pacheco *et al.*, 1993). Translational fusions of the coat protein gene promoter with the *uidA* reporter gene (β-glucuronidase; GUS) were constructed by inserting the GUS-*nos* cassette from

pBI101 into the *Sty*I-digested and blunt-ended pIGV23 (Bevan, 1984; Jefferson *et al.*, 1987). The *Sty*I digestion of pIGV23 eliminates most of the coat protein ORF leaving only the first 7 codons. Three versions (Large, Medium and Small) of the CP promoter were obtained by digesting with *Eco*RI, *Pml*I or *Hin*dIII enzymes and fused to the GUS-*nos* cassette (see Figure 1).

The vectors used for AC2 transactivation assays were constructed as follows: Complementary oligonucleotides containing two CLE sequences (see Figure 3) were synthesized using an Applied Biosystems DNA Synthesizer. The oligonucleotides were designed in such a way that, when annealed, they generated *Hin*dIII (5') and *Xba*I (3') cohesive ends. The oligonucleotides (10 mM each) were annealed essentially as described by Sambrook *et al.* (1989). After annealing the oligonucleotides were inserted upstream of the CaMV 35S-GUS-*nos* fusions found in plasmids pBI46 and pBI90. pBI46 contains the minimal (nucleotides -46/+8-) 35S promoter version, whereas pBI90 contains the truncated (-90/+1) version (Benfey *et al.*, 1990). The newly generated plasmids were named pBI46CLE and pBI90CLE, respectively. These two plasmids were used to obtain transgenic tobacco plants. For transient assays, the CLE-35S (-46 or -90)-GUS-*nos* cassettes were subcloned into pBluescript SK+ to generate the plasmids CLE46GUS and CLE90GUS. To obtain a construct with a single CLE, CLE46GUS and CLE90GUS were digested with *Stu*I and *Hin*dIII (see Figure 3), filled-in and religated generating plasmid 1CLE46GUS and 1CLE90GUS. As bombardment controls, pBluescript SK+ harboring either the minimal or truncated promoters directing the expression of the GUS gene (46GUS and 90GUS, respectively) were used.

To mutate the CLE sequences in the CP promoter, the fragment *Hin*dIII-*Sty*I (SCP; -115 to +19) was synthetically constructed using two complementary oligonucleotides (89 and 87 bases) that overlapped 31 bases and included the mutated CLE sequences. The oligos were annealed, filled-in and ligated into pZero. The mutated *Hin*dIII-*Sty*I fragment was then excised and used to substitute the equivalent wild type fragment in the construct SCP-GUS. The GTGGTCC box found 18 nt upstream the ATG codon was changed to GATTACC. The CLE-like boxes GTGGTC and TTGGTCCC found in inverse orientation at 66 and 81 nt upstream the initiation codon were also changed to GTTTTC and TTTTACCC, respectively.

Two types of constructs were used as a source of AC2 product: plasmid pIGV22 (monomeric infectious clone of PHV A in pBluescript) and a construct where the expression of the AC2 ORF is directed by the 35S CaMV promoter (35S-AC2, also in pBluescript). To confirm AC2-specific effects, a nonsense mutation was introduced into the AC2 ORF of pIGV22 using a site directed mutagenesis kit, following the manufacturers' recommendations (Clontech; Palo Alto, CA). The oligonucleotide used: 5'-CGGCTGTTgAA**TTTAaA**TACATATTAACTG-3', introduced two changes (lower case) that produced two stop codons (underlined) and created a *Dra*I site (boldface). The mutations are located in the segment in which the AC2 ORF does not overlap with either AC3 nor AC1 ORFs.

Transient assays

For transient expression experiments, a particle delivery system (model PDS1000, DuPont) was used to introduce the DNA of the constructs described above into pea or tobacco leaf tissue, following a procedure previously described (Klein *et al.*, 1988). Before the bombardments leaf tissue was incubated for 4 hr at 25 °C on solid MS medium containing 0.4M mannitol and 0.4M sorbitol. The bombardments were carried out at a pressure of 800 lb. per square inch. Bombarded tissue was incubated at 25°C for 18-24 h on solid MS medium without sucrose and then stained for glucuronidase activity (see below). The efficiency of bombardment was determined as the number of blue spots per bombardment per area.

Histochemical staining

Histochemical staining for GUS activity either for transient expression assays or on transgenic tissue was done essentially as described by Jefferson *et al.*, 1987. Tissue was incubated at 37°C for 4-8 h in a solution containing 0.5 mg/ml X-Gluc (Biosynth AG, Switzerland), 50 mM pH 7 phosphate buffer, 10 mM EDTA and 0.1% (V/V) Triton X-100. Stained tissue was cleared of pigments with several 30 min. washes using a 3:1 methanol-acetone mixture, until destaining was complete. The tissue and sections were visualized with a stereoscope (Nikon) or a bright field microscope (Zeiss).

Plant inoculation

Tobacco plants were inoculated via particle bombardment as described before (Garzón-Tiznado *et al.*, 1993). Plants were grown under greenhouse conditions at an average temperature of 28°C.

Plant transformation

Stable transformation of tobacco Xanthi nc plants was carried out by the leaf disc transformation method essentially as described by Horsch and Klee (1986).

A 115 nt minimal PHV CP promoter is transactivated by AC2 protein

In order to delimit the sequences within the PHV CP promoter involved in the transactivation by AC2, the GUS reporter gene was fused to three different versions, in size, of the CP promoter, generating the fusions LCP-GUS (which includes 693 nt upstream from the CP start codon), MCP-GUS (235 nt) and SCP-GUS (115 nt, the first 115 nucleotides of SEQ ID NO: 9) (Figure 1). Transient expression experiments assays showed that all versions of the CP promoter were able to be transactivated by a cloned PHV A component. Then, the shortest construct (SCP-GUS) was introduced into tobacco plants via *Agrobacterium tumefaciens* transformation. Histochemical analysis of primary transformants and T1 plants showed that the expression of the GUS reporter gene is restricted to vascular tissue of segments close to lateral buds and the apical portion of the stem. No GUS activity was detected in leaves or other tissues. These transgenic plants were inoculated via particle bombardment with PHV (Garzón-Tiznado *et al.*, 1993). Leaves and stems were then analyzed for GUS activity 2-3 weeks after inoculation, when symptoms were clearly visible. Inoculated leaves displayed strong glucuronidase activity mostly as discrete points (the sites of impact). In contrast, in mock-inoculated plants no such expression was detectable. These results confirmed that the sequences involved in transactivation by viral factors (most likely AC2) were present in the 115 nt fragment of the CP promoter (SCP).

Conserved Late Element (CLE) sequences are involved in AC2 transactivation

The 115 nt segment contains two elements highly similar to a conserved sequence motif (CLE) found in the promoters of late (i.e. CP and BV1) genes from all geminiviruses from the Old World (OW; Europe, Africa, and Asia) and some from the

New World (NW; America), and postulated as a potential functional target for AC2 (Argüello-Astorga *et al.*, 1994). Figure 2 shows the results of an analysis of the IR from selected OW and NW geminiviruses, in which the relative position and orientation of CLEs (one mismatch allowed) are indicated. In OW geminiviruses, a CLE is always present adjacent to the stem-loop element in both components, although component B generally contains more CLEs. The CLEs found in the NW viruses usually display more variation in their position within the IR.

To define if CLE sequences are indeed involved in the transactivation of PHV late genes, two approaches were followed. First, gain-of-function experiments with heterologous promoters were carried out. Initially, an oligonucleotide 29 bases in length that contains two CLE motifs was synthesized (Figure 3). A *Stu*I site was created between the CLE motifs to facilitate further assays with a promoter containing only one CLE. The synthetic oligonucleotide was cloned upstream of the minimal (nucleotides -46/+8) and truncated (nucleotides -90/+1) versions of the CaMV 35S promoter fused to the GUS reporter gene to generate the plasmids CLE46GUS and CLE90GUS, respectively (Figure 3).

For the transient assays, leaf tissue from a PHV host (tobacco) and a non-host (pea) plant was bombarded with either 46GUS, 90GUS, CLE46GUS or CLE90GUS constructs in the absence or presence of a source of AC2 (either pIGV22 or p35S-AC2). The effect of AC2 was determined as the increase in the number of blue spots (GUS activity) found in the tissues co-bombarded with an AC2 source. As a negative control a PHV AC2 mutant (AC2-) was included in the experiments. Pea and tobacco leaf tissue bombarded with CLE46GUS and CLE90GUS in the absence or the presence of PHV A as a source of AC2 shows in both cases an increased intensity of the number of blue spots in the presence of PHV A DNA. Figures 4A and 4B show graphically the results of the bombardment experiments with tobacco leaves. No transactivation was observed in the experiments with the CLE-less 46GUS or 90GUS DNAs. The number of blue spots obtained was similar irrespective of the accompanying DNA (BS, PHV A, 35S-AC2 or AC2-). On the other hand, the co-bombardment of CLE46GUS (or CLE90GUS) with either PHV A or 35S-AC2 DNAs produced a higher expression of the reporter gene when compared with the controls that included DNA from pBluescript (BS) or the AC2-mutant. Similar results were

obtained with pea tissue, although in this case, a lower background and a higher transactivation level were usually observed. The results obtained clearly show that the 29 nt segment is able to confer AC2-responsiveness to heterologous promoters.

For the second approach, the CLE sequences found in the SCP-GUS construct were modified to disrupt the consensus. Transient experiments comparing the CLE-less SCP-GUS and the wt SCP-GUS showed a similar level of expression in the absence of a AC2 source. However, only the wt SCP-GUS was able to be transactivated when co-bombarded with a source of AC2 (Figure 5).

A single CLE sequence is sufficient to transactivate heterologous promoters

To determine if a single CLE element was sufficient to confer AC2-responsiveness, the CLE46GUS and CLE90GUS constructs were digested with *Stu*I and *Hin*dIII to eliminate 13 nt of the original oligonucleotide including one CLE (see Figure 3). These new constructs, 1CLE46GUS and 1CLE90GUS, were also delivered onto tobacco leaves in the presence or absence of an AC2 source. The results obtained showed that a promoter with a single CLE was still able to be transactivated by AC2 (Figure 4). Again, a lower expression was observed when DNA from pBluescript or the AC2- PHV A mutant were co-bombarded. It was also noticed that the level of transactivation of these constructs with only 16 nt (see also SEQ ID NO: 11) is similar to that obtained with the constructs harboring the complete 29 nt fragment (i.e. two CLEs); see also SEQ ID NO: 10.

The mentioned constructs have been obtained as follows:

Oligonucleotides containing two CLEs motifs (underlined). Two complementary oligos were synthesized and after annealing, they generated two sites in the extremes. The 29 nt sequence is derived from the viral genome (BV1 promoter which is also inducible by AC2 protein. This segment was used from the BV1 promoter only because it contained two CLEs). Only two bases (lower case letters) were changed to introduce a StuI site between the CLE motifs and used to generated the 16 nt oligo shown below.

HindIII cle StuI cle XbaI

AGCTTT**AAA**GTGGTCCC**AAAGGcCtT**GTGGTCCC**A**AAT

AA**TTTCACCAGGGTTTCCgGaACACCAGGGT**TTACTAG

Oligonucleotide containing only one CLE. This oligo was obtained after digestion of the 29 nt oligo with StuI and XbaI (product is 16 nt long). Shown here is as double stranded DNA. The CLE motif is underlined.

StuI cle XbaI

-- AGG CCTTGTGGTCCCAAAT

-- TCC GGAACACCAGGGTTTACTAG

CLE sequence of PHV can also be induced by other wt geminiviruses

Tissues were bombarded with a construct containing the chimeric promoter including the CLE and the minimal 35S promoter (CLE46GUS). This construct was co-bombarded with two versions of the component A of PHV (pIGV23 and pIGV22). Both transactivations were compared with a control wherein pBluescript DNA was used instead of viral DNA. In addition activation of CLE sequence by a wide range of distantly related geminiviruses, including viruses of the Curto-and the Begomovirus genera was performed – these viruses include Taino Tomato Mottle Virus, TPV, ACMV and BCTV. In these experiments the tissue was cobombarded with the CLE46GUS construct and genomic or DNA A clones of the respective viruses. Results are shown in table 1.

It appears that the activity of Texas Pepper geminivirus (recently renamed Pepper Golden Mosaic Virus) is similar to the transactivation of PHV to the CLE of its own species (see table 1). Also the use of Taino tomato mottle virus results in a comparable transactivation of the chimeric promoter as does BCTV (distantly related to PHV).

Table 1

DNA cobombarded with CLE46GUS	*Relative level of expression*
pBluescript (control)	1
PHV (PIGV23)	2
PHV (pIGV 22)	3
TPV	1-2
Taino Tomato mottle virus	2
ACMV	3
BCTV	3

The numbers indicate the relative quantity of blue spots observed on the leaves in the assay used as described above after standardizing background level with pBluescript to 1.

Combination of Silencer plus the CLE elements:

A construct containing two CLE elements coupled to the minimum 35S promoter was engineered to substitute the 35S promoter in a plant transformation vector pTHW136 between the Xbal and Sma I site to give plasmid pTCM. All DNA techniques were carried out according to standard procedures (Sambrook et al., 1989). The CLE and minimum 35S promoter elements were chemically synthesized using two complementary primers and subsequent annealing and polymerization with Klenow DNA polymerase (Biolabs) in the case of the minimum promoter fragment. The primer sequence were as follows:

Cle1: gtggatcctttgggaccacaaggcctttgggaccactttaactagtcg (SEQ ID NO: 12)

Cle2: cgactagttaaagtggtcccaaaggccttgtggtcccaaaggatccac (SEQ ID NO: 13)

Min-r: ccggatcccaagacccttcctctatataag (SEQ ID NO: 14)

Min-d: ggcccgggttcagcgtgtcctctccaaatgaaatgaacttccttatatagag (SEQ ID NO: 15)

The sequence of TGMV (Accession. No. K02829) and PHV DNA-A (Accession. No. X70419) were obtained from the EMBL database. The sequences were aligned using the GCG program from the Wisconsin Package. The silencer-like region in the PHV DNA genome (Ruiz-Medrano et al. 1999) was narrowed by comparison to the

silencer-like element from TGMV (Sunter and Bisaro 1997). Two primers were used to amplify the PHV silencer:

Sil-1: cccaagcttctccactagccgtattttg (SEQ ID NO: 16)

Sil-2: gcgcgtcgacttcctataaagactacctca (SEQ ID NO: 17)

These contained a HindIII site and a Sal I site to facilitate cloning.

The amplified DNA fragment was digested with Hind III and SalI and subcloned in pTCM between the Hind III and the Sal I site to give pTCMS. Thus the silencer-like element is placed downstream of the GUS coding region but within the left and right borders of Agrobacterium. It should be noted that the silencer elements can also be placed upstream of the GUS coding region.

Using methods known to the person skilled in the art additional constructs can be made using similar procedures to replace the GUS open reading frame with sequences of interest.

GUS activity of pTCM and pTCMS can be assayed in leaf discs using transient assays according to procedures well known to those skilled in the art. In the case of heterologous genes of interested intended to affect viral replication the constructs can be assayed in transient assays by co-inoculating the construct with viral DNA clones and measuring the newly viral replicated DNA on southern blots. The constructs can be introduced in plants via agrobacterium tumefaciens for instance, and the plants will be analyzed for GUS expression and/or viral resistance using techniques well known to those skilled in the art.

References

Abouzid A.M., Frischmuth T, Jeske H (1988). A putative replicative form of the abutilon mosaic virus (gemini group) in a chromatin-like structure. Molecular and General Genetics, **212,** 252-258.

Argüello-Astorga, G., Herrera-Estrella, L., and Rivera-Bustamante, R. (1994a). Experimental and theoretical definition of geminivirus origin of replication. *Plant Molecular Biology* **26**, 553-556.

Argüello-Astorga, G.R., Guevara-González, R.G., Herrera-Estrella, L.R., and Rivera-Bustamante, R.F. (1994b). Geminivirus replication origins have a group-specific organization of iterative elements: A model for replication. *Virology* **203**, 90-100.

Argüello-Astorga, G., Herrera-Estrella, L. (1995). Theoretical and experimental definition of minimal photoresponsive elements in *cab* and *rbcS* genes. In: Terzi M, Cella R and Falavigna A (eds). Current issues in plant molecular and cellular biology. Kluwer Academic Publishers, Dordrecht, The Netherlands., pp. 501-511.

Benfey, P.N., Ren, L., and Chua, N.H. (1990). Combinatorial and synergistic properties of CaMV 35S enhancer subdomains. *The EMBO Journal* **9**, 1685-1696.

Bevan, M.W. (1984). Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Research* **12**, 8711-8720.

Briddon R.W, Bedford I.D, Tsai J.H, Markman P.G (1996). Analysis of the nucleotide sequence of the treehopper-transmitted geminivirus, tomato pseudo-curly top virus, suggests a recombinant origin. Virology, **219**: 387-394.

Bisaro DM (1998). Gene Regulation in Geminivirus. Abstract at 2nd International Workshop on Bemisia and Geminiviral Diseases. Puerto Rico 7-12 June 1998.

Cramer CL; Weissenborn DL; Oishi KK; Grabau EA; Bennett S; Ponce E; Grabowski GA; Radin DN (1996). Bioproduction of human enzymes in transgenic tobacco. Ann. NY Acad. Sci., 792():62-7.

Davies, J.W., and Stanley, J. (1989). Geminivirus genes and vectors. *Trends in Genetics* **3**(3), 77-81.

Davies *et al.*, J. Cell. Sci (Suppl.) (1987) 7: 95-107

Day AG; Bejarano ER; Buck KW; Burrell M; Lichtenstein CP (1991). Expression of an antisense viral gene in transgenic tobacco confers resistance to the DNA virus tomato golden mosaic virus. Proc. Natl. Acad. Sci. USA, 88(15):6721-5

Frischmuth T and Stanley J (1998). Recombination between viral DNA and the transgenic coat protein gene of African cassava mosaic geminivirus. J. General Virology 79, 1265-1271.

Garzón-Tiznado, J.A., Torres-Pacheco, I., Ascencio-Ibañez, J.T., Herrera-Estrella, L., and Rivera-Bustamante, R.F. (1993). Inoculation of peppers with infectious clones of a new geminivirus by a biolistic procedure. *Phytopathology* **83**(5), 514-521.

Gröning BR; Hayes RJ; Buck KW (1994). Simultaneous regulation of tomato golden mosaic virus coat protein and AL1 gene expression: expression of the AL4 gene may contribute to suppression of the AL1 gene. J. Gen. Virol., 75 (Pt 4)():721-6.

Hahn S.K., Terry, E.R. and Leuschner, K. (1980). Breeding cassava for resistance to cassava mosaic disease. Euphytica 29, 673-683.

Haley, A., Zhan, X., Richardson, K., Head, K., and Morris, B. (1992). Regulation of the activities of African cassava mosaic virus promoters by the AC1, AC2 and AC3 gene products. *Virology* **188**(2), 905-909.

Hong, Y.G., Robinson, D.J., and Harrison, B.D. (1993). Nucleotide sequence evidence for the occurrence of three distinct whitefly-transmitted geminiviruses in cassava. *Journal of General Virology* **74**(11), 2437-2443.

Hong Y; Saunders K; Hartley MR; Stanley J (1996). Resistance to geminivirus infection by virus-induced expression of dianthin in transgenic plants. Virology, 220(1):119-27

Hormuzdi, S.G and Bisaro D.M. (1995). Genetic analysis of beet curly top virus. Examination of the roles of L2 and L3 genes in viral pathogenesis. Virology 206, 1044-1054.

Horsch, R.B., and Klee, H. (1986). Rapid assay of foreign gene expression in leaf discs transformed by *Agrobacterium tumefaciens*: Role of T-DNA borders in the transfer process. *Proceedings of the National Academy of Sciences of the USA* **83**, 4428-4432.

Jefferson, R.A., Kavanaugh, T.A., and Bevan, M.W. (1987). GUS fusions: ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO Journal* **6**, 3901-3907.

Karkashian JP and Maxwell DP (1998). Molecular analysis of the coat protein gene promoter of Bean Golden Mosaic Gemini virus. Abstract at 2nd International Workshop on Bemisia and Geminiviral Diseases. Puerto Rico, 7-12 June 1998.

Klein, T.M., Fromm, M.E., Weisinger, A., Tomes, A., Schaaf, D., Sletten, M., and Sanford, J.C. (1988). Transfer of foreign genes into intact maize cells using high-velocity microprojectiles. *Proceedings of the National Academy of Sciences of the USA* **85**, 4305-4309.

Kosugi, S., Suzuka, I., and Ohashi, Y. (1995). Two of three promoter elements identified in a rice gene for proliferating cell nuclear antigen are essential for meristematic tissue-specific expression. *The Plant Journal* **7**, 877-886.

Lazarowitz, S.G. (1992). Geminiviruses: Genome structure and gene function. *Critical Reviews in Plant Sciences* **11**(4), 327-349.

Ma JK; Hein MB. (1996) Antibody production and engineering in plants. Ann. NY Acad. Sci., 792():72-81.

Martin, K.J., and Green, M.R. (1992). Transcriptional activation by viral immediate early proteins: Variations on a common theme. In "Transcriptional Regulation" (S. L. McKnight, and K. R. Yamamoto, Eds.), pp. 695-725. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, USA.

Mühlbach HP. (1998). Use of plant cell cultures in biotechnology. Biotechnol Annu. Rev., 4():113-76.

Nevins, J.R. (1991). Transcriptional activation by viral regulatory proteins. *Trends in Biochemical Sciences* **16**, 435-439.

Noris E, Jupin I, Accotto G.P, Gronenborn B. (1996). DNA binding activity of the C2 protein of tomato yellow leaf curl geminivirus. Virology, **217**,607-612.

Noris E; Accotto GP; Tavazza R; Brunetti A; Crespi S; Tavazza M. (1996) Resistance to tomato yellow leaf curl geminivirus in Nicotiana benthamiana plants transformed with a truncated viral C1 gene. Virology, 224(1):130-8.

Petty IT, Coutts RM, Buch KW, (1986), Geminivirus coat protecting gene promoter sequences can function in Escherichia coli, Nucleic Acids Res. 14(12): 5113.

Rigden JE, Dry IB, Krake LR, Rezaian MA, (1996), Plant virus replication processes in Agrobacterium; insight into the origins of geminiviruses? PNAS 93: 10280-4.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). "Molecular Cloning. A laboratory manual." 2nd Ed. 3 vols. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Stanley J; Latham JR; Pinner MS; Bedford I; Markham PG (1992). Mutational analysis of the monopartite geminivirus beet curly top virus. Virology, 191(1):396-405.

Sung Y.K, and Coutts R.H (1996). Potato yellow mosaic geminivirus AC2 protein is a sequence non-specific DNA binding protein. FEBS Letters, **383,** 51-54.

Sunter, G., and Bisaro, D.M. (1991). Transactivation of a geminivirus: AL2 gene product is needed for coat protein expression. *Virology* **180**, 416-419.

Sunter, G., and Bisaro, D.M. (1992). Transactivation in geminivirus AR1 and BR1 gene expression by the viral AL2 gene product occurs at the level of transcription. *Plant Cell* 4(10), 1321-1331.

Sunter, G., and Bisaro, D.M. (1997). Regulation of a geminivirus coat protein by AL2 (TrAP): evidence for activation and derepression mechanisms. *Virology*, **232** (2), 269-280.

Sunter, G., and Bisaro, D.M. (1997b) Regulation of Gene Expression. Abstract 245: The American Society of Plant Physiologists, Aug 6 1997.

Sunter, G., Hartitz, M.D., and Bisaro, D.M. (1993). Tomato golden mosaic virus leftward gene expression: Autoregulation of geminivirus replication protein. *Virology* **195**(1), 275-280.

Sunter, G., Stenger, D.C., and Bisaro, D.M. (1994). Heterologous complementation by geminivirus AL2 and AL3 genes. *Virology* **203**(2), 203-210.

Timmermans, M.C.P., Prem Das, O., and Messing, J. (1994). Geminiviruses and their uses as extrachromosomal replicons. *Annual Review of Plant Physiology and Plant Molecular Biology* **45**, 79-112.

Torres-Pacheco, I., Garzón-Tiznado, J.A., Herrera-Estrella, L., and Rivera-Bustamante, R.F. (1993). Complete nucleotide sequence of pepper huasteco virus:

Analysis and comparison with bipartite geminiviruses. *Journal of General Virology* **74**, 2225-2231.

Vancanneyt G; Schmidt R; O'Connor-Sanchez A; Willmitzer L; Rocha-Sosa M (1990). Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in Agrobacterium-mediated plant transformation. Mol. Gen. Genet, 220(2):245-50.

Wartig L; Kheyr-Pour A; Noris E; De Kouchkovsky F; Jouanneau F; Gronenborn B; Jupin I (1997). Genetic analysis of the monopartite tomato yellow leaf curl geminivirus: roles of V1, V2, and C2 ORFs in viral pathogenesis. Virology, 228(2):132-40.

CLAIMS

1. A chimeric promoter capable of mediating gene expression of a heterologous DNA sequence in plants upon geminivirus infection comprising
   (i) at least one cis-acting element sufficient to confer geminivirus-inducible expression comprising the nucleotide sequence of any one of SEQ ID NOS.: 1 to 11 or a functionally equivalent nucleotide sequence; and
   (ii) a promoter.

2. The chimeric promoter of claim 1, comprising at least one cis-acting element having the nucleotide sequence of SEQ ID NO: 2.

3. The chimeric promoter of claim 1 or 2, wherein said chimeric promoter comprises homo- and/or hetero-multimeric forms of said cis-acting element(s).

4. The chimeric promoter of any one of claims 1 to 3, wherein said multimeric form is a dimer.

5. The chimeric promoter of any one of claims 1 to 4, wherein the promoter is a truncated or minimal promoter.

6. The chimeric promoter of any one of claims 1 to 4, wherein the truncated or minimal promoter is derived from the CaMV35S promoter, CHS promoter, PR1 promoter, nos promoter, Ac promoter, Adh-1 promoter, Bz1 promoter or LAT52 promoter.

7. The chimeric promoter of any one of claims 1 to 6, wherein the distance between said cis-acting element and the transcription start site of the promoter is 8 to 100 base pairs.

8. A recombinant gene comprising the chimeric promoter of any one of claims 1 to 7.

9. The recombinant gene of claim 8 further comprising a silencer-like element.

10. The recombinant gene of claim 9, wherein said silencer-like element is derived from the genome of a geminivirus.

11. The recombinant gene of claim 10, wherein said silencer-like element is located around nucleotide 2150 of the PHV genome or functionally similar elements in other geminiviruses in the same relative position.

12. The recombinant gene of any one of claims 8 to 11, wherein the chimeric promoter is operatively linked to a heterologous DNA sequence.

13. The recombinant gene of claim 12, wherein at least one of said cis-acting or silencer-like elements is located in the 5'- or 3-untranslated region or in an intron of the recombinant gene.

14. The chimeric promoter of any one of claims 1 to 7 or the recombinant gene of any one of claims 8 to 13, wherein said chimeric promoter or recombinant gene is expressible when the cell is either infected with a geminivirus or part thereof of the same species or is infected with a geminivirus or part thereof or another species.

15. The recombinant gene of any one of claims 9 to 14, wherein said heterologous DNA sequence encodes a (poly)peptide, cytotoxic protein, antibody, antisense RNA, sense RNA, ribozyme, transcription factor or a suicide gene.

16. The recombinant gene of any one of claims 12 to 15, wherein expression of the heterologous DNA sequence in a plant cell results in a plant with reduced susceptibility to a geminiviral infection and spread thereof.

17. A vector comprising the chimeric promoter of any one of claims 1 to 7 or the recombinant gene of any one of claims 8 to 16.

18. A method for the production of a transgenic plant with a reduced susceptibility to a geminiviral infection and spread thereof comprising the introduction of a

recombinant gene of any one of claims 8 to 16 or the vector of claim 17 into the genome of a plant, plant cell or plant tissue.

19. Plant cells comprising a chimeric promoter of any one of claims 1 to 7, the recombinant gene of any one of claims 8 to 16 or the vector of claim 17 or obtainable by the method of claim 18.

20. A transgenic plant or plant tissue comprising plant cells of claim 19.

21. The transgenic plant of claim 20, which upon the presence of the chimeric promoter or the recombinant gene attained resistance or improved resistance against a geminiviral infection the corresponding wild-type plant was susceptible to.

22. Harvestable parts or propagation material of a transgenic plant of claim 20 or 21 comprising plant cells of claim 19.

23. The harvestable parts or propagation material of claim 22 which are leaves, flower, seed, seedlings, roots, pollen or tubers.

24. A kit comprising a chimeric promoter of any one of claims 1 to 7, the recombinant gene of any one of claims 8 to 16 or the vector of claim 17.

25. Use of a cis-acting element as defined in any one of claims 1 to 7, a chimeric promoter of any one of claims 1 to 7, the recombinant gene of any one of claims 8 to 16 or the vector of claim 17 for the production of geminivirus resistant plants.

26. Use of a cis-acting element as defined in any one of claims 1 to 7, the chimeric promoter of any one of claims 1 to 7, a recombinant gene of any one of claims 8 to 16 or the vector of claim 17, to specifically arrest the spread or outgrowth of geminivirus in an infected plant or plant cell(s) as a consequence of killing or damaging the geminiviral infected cell(s).

27. A method of rendering a gene responsive to geminivirus infection comprising inserting at least one cis-acting element as defined in any one of claims 1 to 7 into the promoter of said gene.

28. A method for preparing a promoter capable of mediating gene expression in plants upon geminivirus infection comprising operably linking a cis-acting element as defined in any one of claims 1 to 8 to a transcription initiation sequence of a promoter.

29. The method of claim 27 or 28, further comprising deleting non-specific cis-acting elements in the promoter.

30. The promoter obtainable by the method of any one of claims 27 to 29.

31. A method for preventing or diminishing geminivirus infection in a plant and subsequent outgrowth of said virus comprising planting a plant of claim 20 or 21 in an area susceptible to geminiviral infection.

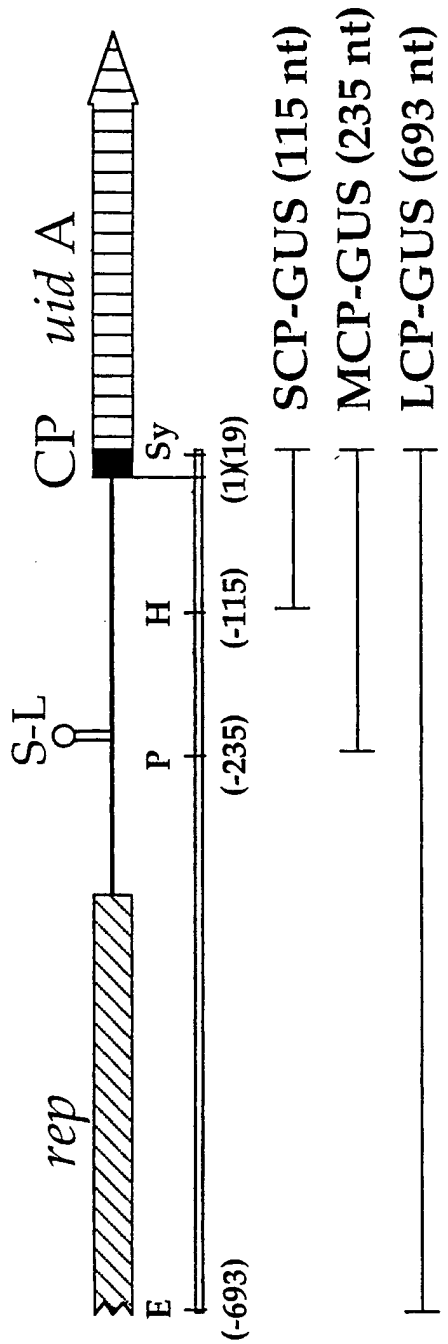
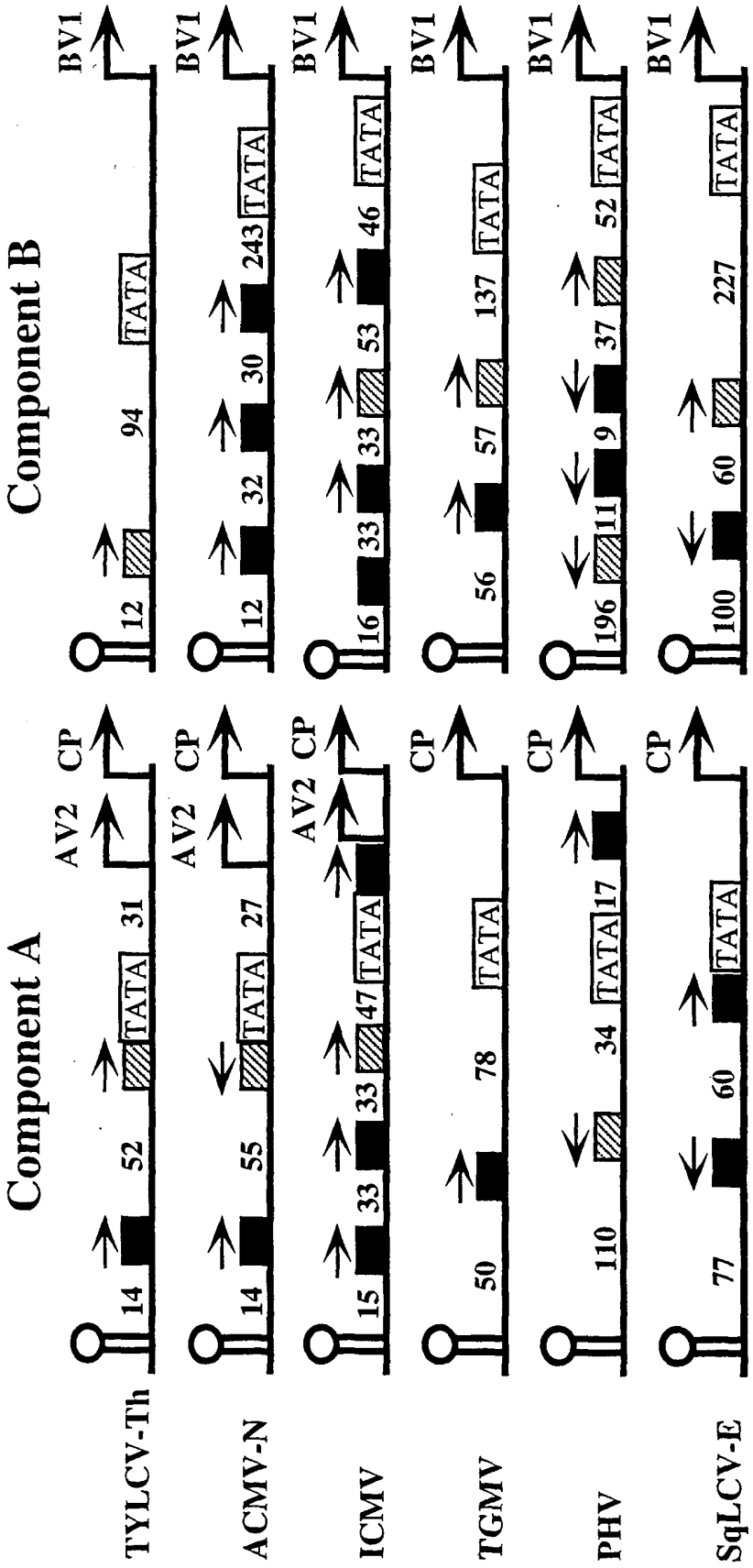


FIGURE 1



CLE: ■ Complete consensus GTGGTCCC ▨ Incomplete consensus (1 mismatch)

Figure 2

Figure 3

Fig. 4A

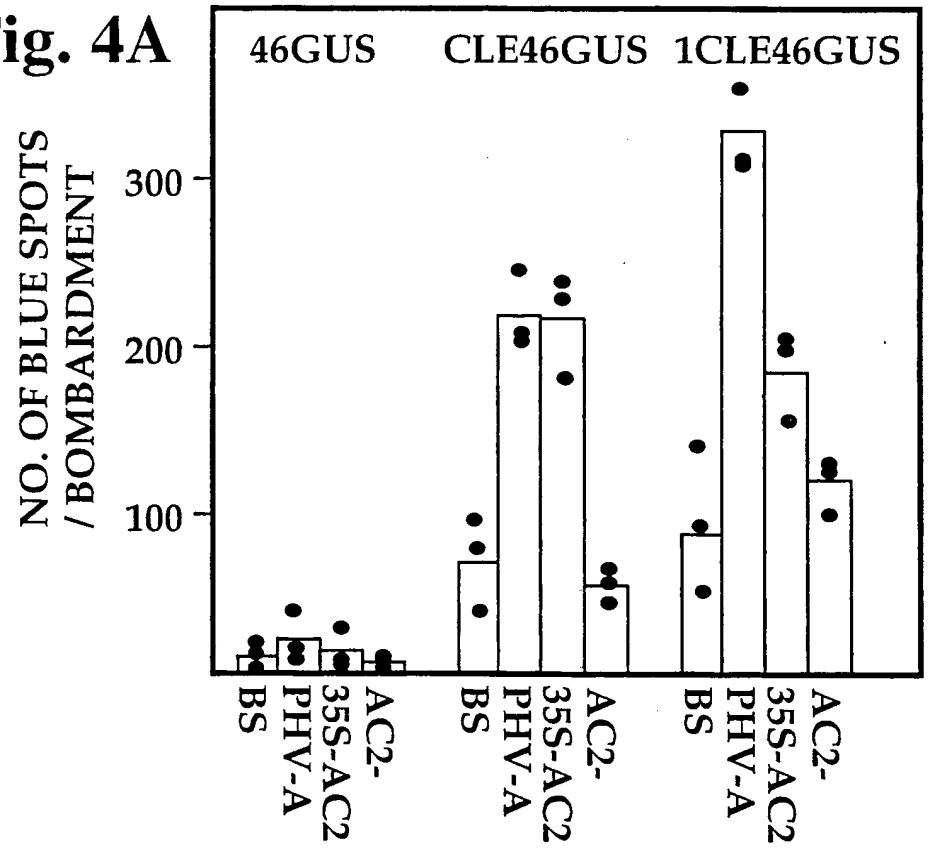
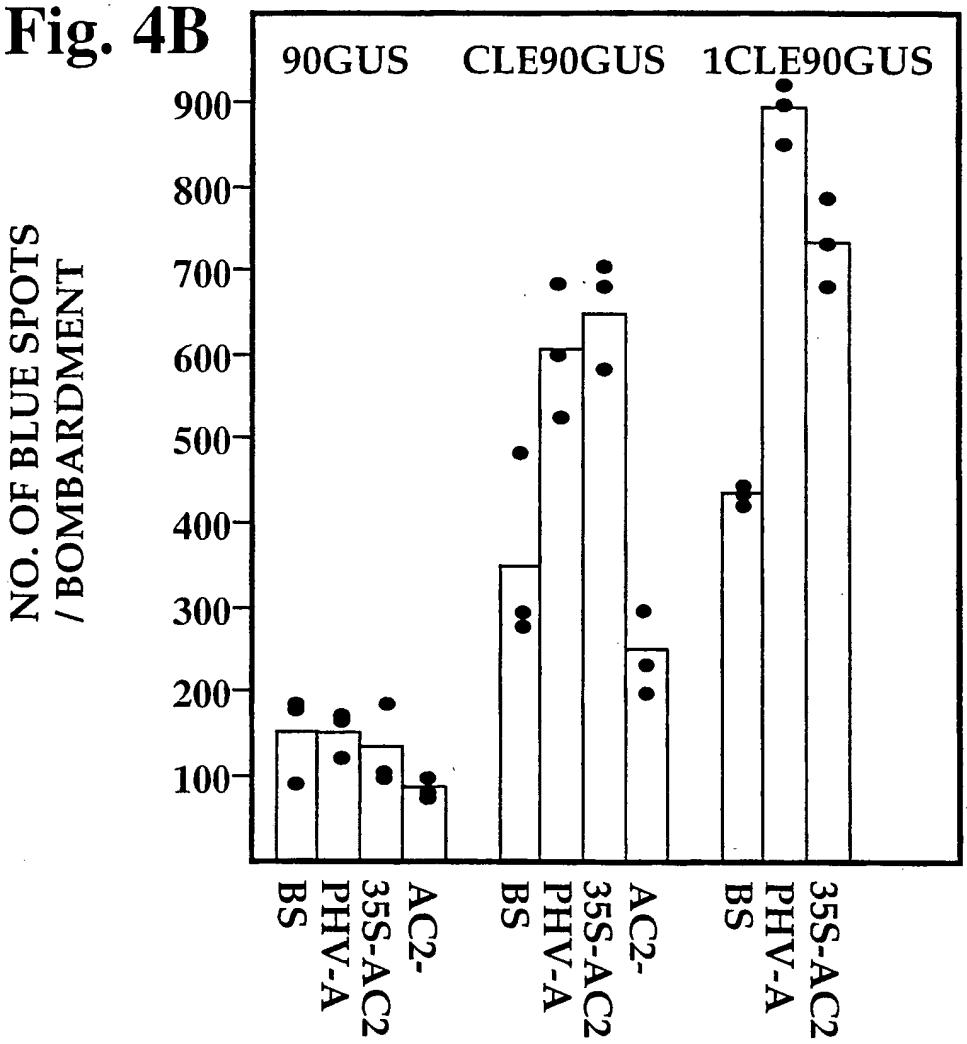


Fig. 4B



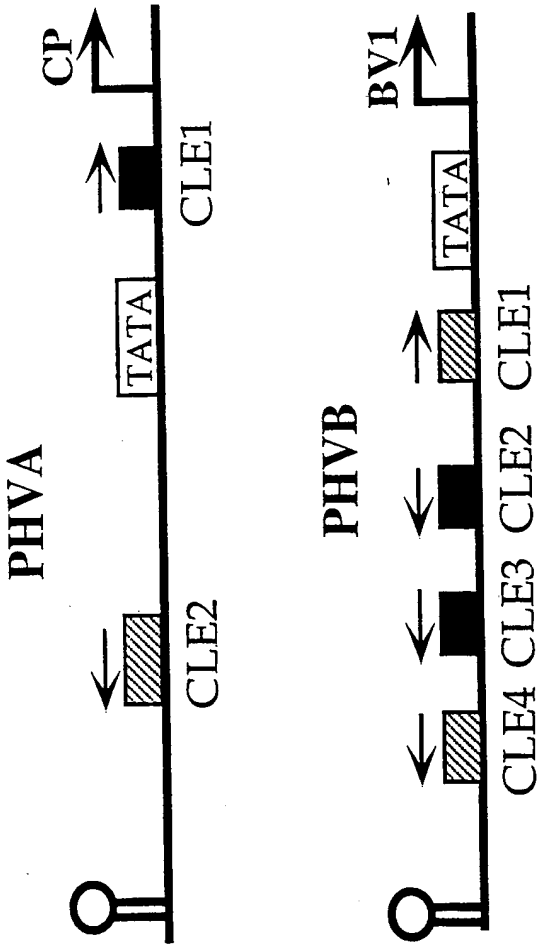


Fig. 5A

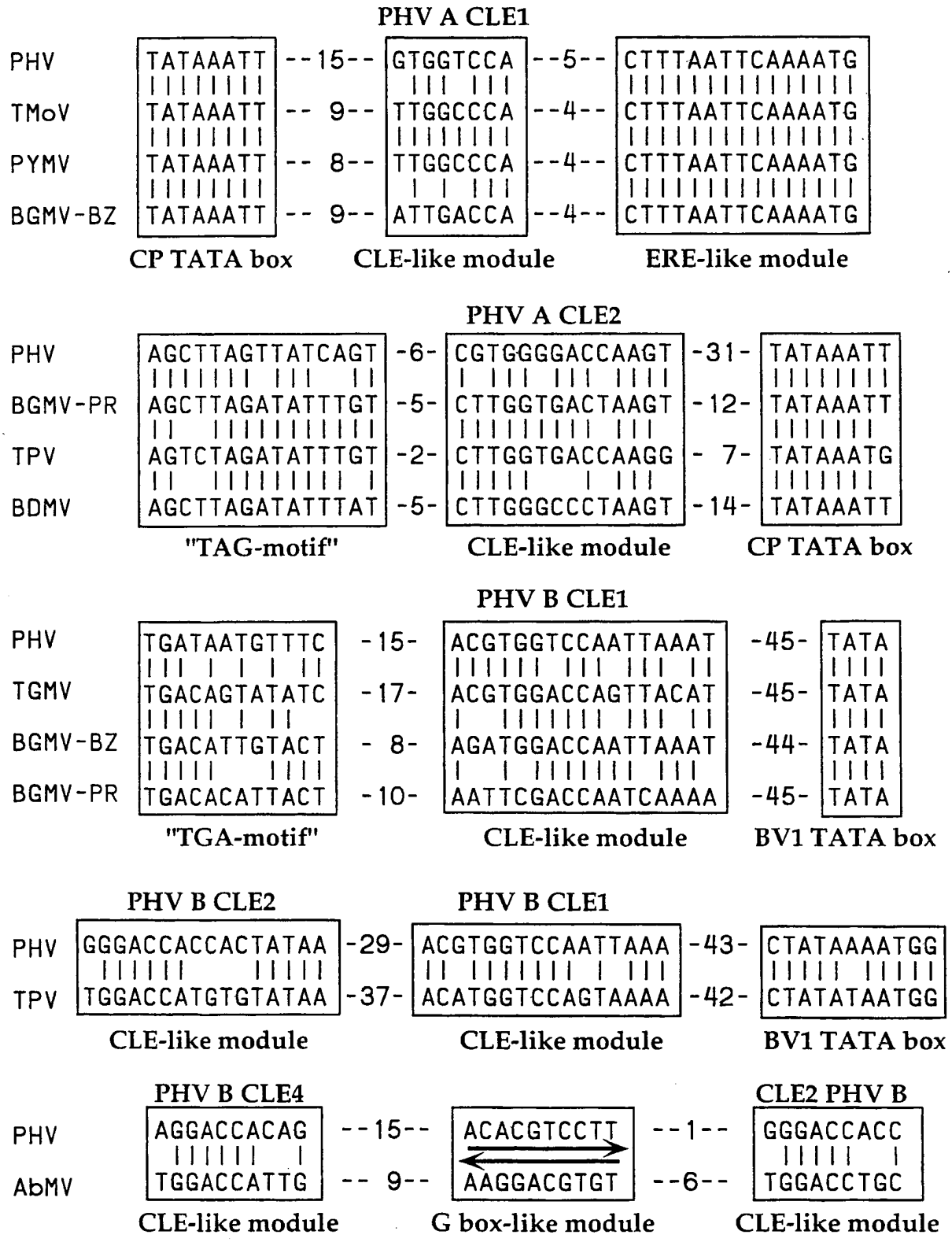


Fig. 5B

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: CINVESTAV
(B) STREET: Apdo. Postal 629
(C) CITY: Irapuato
(E) COUNTRY: Mexico
(F) POSTAL CODE (ZIP): 36500

(ii) TITLE OF INVENTION: Geminivirus inducible promoter sequences and the uses thereof

(iii) NUMBER OF SEQUENCES: 17

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

RWGTGGTCCC 10

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GTGGTCC 7

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GTGGTCCA 8

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TTGGCCCA 8

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TTGGTCCC 8

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GTGGACCA 8

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GACCAC 6

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GGGACCAA 8

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GAAGCTTAGT TATCAGTTCC AGACGTGGGG ACCAAGTAGT GTATGACCAC TTTATTGACT 60

GTCAGCTTTA TAAATTGAAA TTAAAACATA AGTGGTCCAT GTACCTTTAA TTCAAAATG 119

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AGCTTTAAAG TGGTCCCAAA GGCCTTGTGG TCCCAAATGA TC 42

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

AGGCCTTGTG GTCCCAAATG ATC 23

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GTGGATCCTT TGGGACCACA AGGCCTTTGG GACCACTTTA ACTAGTCG 48

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CGACTAGTTA AAGTGGTCCC AAAGGCCTTG TGGTCCCAAA GGATCCAC 48

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CCGGATCCCA AGACCCTTCC TCTATATAAG 30

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GGCCCGGGTT CAGCGTGTCC TCTCCAAATG AAATGAACTT CCTTATATAG AG 52

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CCCAAGCTTC TCCACTAGCC GTATTTTG 28

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GCGCGTCGAC TTCCTATAAA GACTACCTCA 30

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/82, 15/33, 5/10, A01H 5/00

A3

(11) International Publication Number: WO 99/60140

(43) International Publication Date: 25 November 1999 (25.11.99)

(21) International Application Number: PCT/IB99/01282

(22) International Filing Date: 19 May 1999 (19.05.99)

(30) Priority Data:
98201636.2 19 May 1998 (19.05.98) EP

(71) Applicant *(for all designated States except US)*: CENTRO DE INVESTIGACION Y DE ESTUDIOS AVANZADOS DEL I.P.N. (CINVESTAV) [MX/MX]; Departamento de Ingeniería Genética, CP 629, Irapuato, Guanajuato 36500 (MX).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: RIVERA–BUSTAMANTE, Rafael, F. [MX/MX]; Infiernillo 1231, Colonia CFE, Irapuato, Guanajuato 36631 (MX). RUIZ–MEDRANO, Roberto [MX/MX]; Matavacas 5 San Javier, Guanajuato, Guanajuato 36020 (MX). ARGÜELLO–ASTORGA, Gerardo [MX/MX]; Rio Tamesi 1561, Colonia La Pradera, Irapuato, Guanajuato 36630 (MX). MONSALVE–FONNEGRA, Zulma I. [CO/CO]; Calle 49 #46 A100, Barrio San Francisco, Copacabana, Antioquia (CO).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(88) Date of publication of the international search report:
15 June 2000 (15.06.00)

(54) Title: GEMINIVIRUS INDUCIBLE PROMOTER SEQUENCES AND THE USES THEREOF

(57) Abstract

Novel chimeric promoters which allow controlled transcription and/or expression of a nucleic acid sequence upon geminivirus infection, and the use of such recombinant promoters are provided. Furthermore, recombinant genes comprising such promoters and transgenic plant cells and plants comprising the chimeric promoters or recombinant genes are described.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/IB 99/01282

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N15/33 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SUNTER, G. AND BISARO, D.M.: "regulation of a geminivirus coat protein promoter by AL2 protein (TrAP): evidence for activation and derepression mechanisms" VIROLOGY, vol. 232, 1997, pages 269-280, XP002102486 cited in the application page 269, left column; page 270; page 271, right column; page 274, left column; page 275, right column; page 277, page 277,278	1-31

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 March 2000	13/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Holtorf, S

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/IB 99/01282

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	HONG Y ET AL: "Resistance to geminivirus infection by virus-induced expression of dianthin in transgenic plants" VIROLOGY, vol. 220, no. 12, 1 June 1996 (1996-06-01), pages 119-127, XP002093971 the whole document	1-31
A	WO 94 19477 A (CALGENE INC ;UNIV CALIFORNIA (US)) 1 September 1994 (1994-09-01) page 4, line 25-38; page 19, line 11-17	1-31
A	TORRES-PACHECO, I., ET AL.: "complete nucleotide sequence of pepper huasteco virus: analysis and comparison with bipartite geminiviruses" JOURNAL OF GENERAL VIROLOGY, vol. 74, 1993, pages 2225-2231, XP002102488 cited in the application abstract; fig. 1	1-31
A	ARGÜELLO-ASTORGA, G.R., ET AL.: "geminivirus replication origins have a group-specific organization of iterative elements: a model for replication" VIROLOGY, vol. 203, 1994, pages 90-100, XP002102487 cited in the application page 95; figure 5	1-31
P,X	RUIZ-MEDRANO, R., ET AL.: "identification of a sequence element involved in AC2-mediated transactivation of the peper huasteco virus coat protein gene" VIROLOGY, vol. 253, January 1999 (1999-01), pages 162-169, XP002102489 the whole document	1-8,12, 14,15, 17, 19-23, 27-30

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inten nal Application No
PCT/IB 99/01282

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9419477 A	01-09-1994	US 5650303 A	22-07-1997
		CA 2156720 A	01-09-1994
		EP 0686197 A	13-12-1995
		JP 8509860 T	22-10-1996
		US 5589379 A	31-12-1996
		US 5981236 A	09-11-1999